JOURNAL OF THE
NATIONAL CANCER INSTITUTE

VOLUME 6 *October 1945* NUMBER 2

COLCHICINE IN THE EXPERIMENTAL CHEMOTHERAPY OF CANCER

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I. MODE OF ACTION

INTRODUCTION

Colchicine is an alkaloid present in the corm, seeds, and flowers of the autumn crocus, *Colchicum autumnale*. According to Sharp (*1*), the word "colchicum" is derived from Colchis, the former name of a district of Asia Minor, where several species of the plant grew. The origin of the use of colchicum preparations for the treatment of gout is uncertain, but it has been attributed to the Byzantines (Lits (*2*)). The alkaloid, colchicine, was not isolated until the nineteenth century. Only within the last decade has its remarkable action on cell division been elucidated and found such wide application in biologic and medical research. The modern era of cytologic research opened with the work of Amoroso, in London, and of Lits, in Brussels, although Dixon appears to have been the first to draw attention to the action of colchicine in cell division. In his Manual of Pharmacology, Dixon (*3*) stated that one effect of this drug is "to excite karyokinesis" in the bone marrow. He added further (*3, p. 96*):

> The exact significance of this action on the marrow cannot be adequately determined at present; but it should not be regarded as specific to the leucocytes, but rather a type of the action which goes on to a greater or less degree in other tissues of the body * * *.

Amoroso (*4*) saw in this suggestion of Dixon a possible explanation of his clinical experiences in Dublin in 1927, when he was working with the late Prof. M. R. J. Hayes. Together they had observed the "beneficial effects of deep X-ray therapy on neoplasms in patients suffering from acute attacks of gout, which were being treated with colchicum" (*4, p. 266*). They conceived the possibility that colchicine had stimulated mitosis in the malignant cells and consequently rendered them more sensitive to irradiation.

In the first paper on colchicine from the Brussels laboratory, Lits (*5*) reported that its injection into mice was followed by "une véritable explosion de mitoses" in almost every tissue. He pointed out that its action resembled that of a class of substances of varied chemical constitution, which Dustin (*6*) called "poisons caryoclasiques" (mitotic poisons), because of their influence on cell division in the animal body. The theoretical significance which Dustin attributed to these substances, together with the relevant literature up to 1937, was reviewed by Chodkowski (*7*).

ACTION OF COLCHICINE ON CELL DIVISION

Colchicine is the most potent of mitotic poisons, being active in vitro in a dilution of 1 part in 100,000,000 (Ludford (*8*)). In order to account for the vast numbers of dividing cells seen after the injection of colchicine into tumor-bearing animals, Dustin (*9*) was led to conclude that it first stimulated cell division and then arrested it at the metaphase in both normal and malignant cells. He described colchicine as 'le réactif idéal de l'état de capacité divi-

sionelle" of cells, or of their "imminence caryocinétique." Following a single injection of 0.025 mg. of the drug into tumor-bearing mice, Dustin (*10*) reported the maximum number of arrested mitoses about the ninth hour, a diminution in numbers after 24 hours, and an approach towards the normal number after 48 hours. According to the extent of the toxic action on the cells, they either became necrotic or recovered sufficiently to complete division. Ludford (*8*) showed that if a second injection of colchicine is given before the cells arrested in mitoses have been able to complete division, then the action is cumulative. Tumors from animals which have received repeated injections exhibit an enormous number of arrested mitotic figures. In some areas there are more cells held up in division than there are "resting" cells.

Ludford (*8*) introduced the tissue-culture method for investigating the nature of this mitotic poisoning. Embryonic and malignant tissues were grown together in cover-clip cultures, and colchicine was applied to the cultures either at the time of their preparation or a day or two after growth had commenced. It was found that the drug had the same action upon cell division in cultures as in the animal body, which demonstrated unequivocally that the action was a direct one upon the cells. There was no difference between the sensitivity of dividing embryonic and malignant cells to the mitotic poisoning. The accumulation of arrested mitoses was ascribed to failure of spindle formation. Chromosomes were formed, split ready to be separated, but the spindle required to separate them into two groups could not be seen, either in living cultures or after various fixing and staining methods had been employed. This condition was attributed to a lowering of cytoplasmic viscosity preventing the coagulation of the spindle substance. The chromosomes became distorted with increase in concentration of the drug. In extreme cases they shrank to form granular bodies or coalesced to form a single rounded mass of chromatin. Typically, the chromosomes of an arrested mitosis are clustered together in the center of the cell, but in the cells of some tumors they tend to become scattered peripherally.

The property of inhibiting spindle formation is not a specific attribute of colchicine. There is a spontaneously occurring mitotic aberration in some tumors which is characterized by the chromosomes being formed in the normal manner without any spindle being developed (Ludford (*11*). Then other chemical as well as physical agents are capable of blocking mitosis by inhibiting spindle formation, but they invariably prevent the onset of mitosis. Colchicine is unique in that it does not arrest the initial phase of division but brings the process to a standstill at the metaphase, in a remarkably wide range of concentrations.

That the specific cytologic action of colchicine is to inhibit the formation of mitotic spindle has been confirmed by all subsequent investigations. By employing the centrifuge technique, both Beams and Evans (*12, 13*) and Wilbur (*14*) found that the increase in viscosity which normally follows fertilization in sea urchins' eggs (Arbacia) is inhibited by colchicine and no spindles are formed. Beams and Evans also reported that when the drug was applied to eggs in the early stages of division it induced a reversal of the asters and spindle to the sol state. Paletta and Cowdry (*15*), who applied a centrifugal force of 300,000 times gravity to mouse-carcinoma cells for 30 minutes, stated that no consistent difference in displaceability of chromosome clumps was observed between colchicinized and uncolchicinized carcinomas, although they could observe no spindles in the former.

Cells arrested in division by colchicine present a characteristic appearance which is sometimes referred to as "colchicinic mitosis." Most commonly cells exhibit "turgescence et aspect clair de cytoplasme (Dustin (*10*)) with the chromosomes clustered in the center. Barber and Callan (*16*) pointed out that during the normal anaphase,

when the chromosomes are being drawn apart into two groups, the dividing cell swells by the intake of fluid and assumes an even rounded outline. In the colchicinic mitosis, the cytoplasm is in a "condition of prolonged and thereby exaggerated anaphase although the chromosomes are still in metaphase" (*16, p. 264*). It therefore remains abnormally swollen. Barber and Callan reported that larvae of the newt, subjected to intensive colchicine treatment, became considerably swollen. They most aptly described this condition as a state of "intracellular dropsy."

Dividing cells which survive the action of colchicine exhibit various abnormalities. Between 12 and 48 hours after a single injection, Brues and Jackson (*17*) reported "bizarre nuclear figures" in regenerating liver cells—large amoeboid-shaped nuclei, and cells with numerous minute nuclei apparently formed by scattered groups of chromosomes. Increased chromosome numbers were also described in animal cells following colchicine treatment (Tennant and Liebow (*18*)). This is of common occurrence in cells of treated plants. It is the result of the chromosomes being divided longitudinally but not being separated into two daughter nuclei owing to the absence of a spindle. So they form a single nucleus with double the normal number of chromosomes. Repetition of the process produces cells with greatly increased chromosome numbers—polyploidy (Dustin, Havas, and Lits (*19*), Blakeslee (*20*)).

Whether colchicine stimulates mitosis as well as blocking it at metaphase (as Dustin believed and with which Ludford disagreed) is of practical importance when considering the possibilities of this alkaloid as a chemotherapeutic agent for cancer. The application of quantitative methods to this problem has failed to reveal any indication of a stimulation of cell division. Brues (*21*), employing regenerating-rat-liver cells following partial hepatectomy, Bucher (*22*), using rabbit fibroblasts in tissue cultures, and Tennant and Liebow (*18*), using mouse carcinoma also in vitro, could find no indication that colchicine stimulated cell division. On the contrary, Tennant and Liebow reported that in dilutions as low as 1 part in 64,000,000 the drug diminished the rate at which cells entered mitosis although the cultures exhibited a remarkable accumulation of arrested metaphases.

It has been pointed out that the results of mitotic poisoning are most obvious in tissues where mitosis is most frequent, and consequently colchicine has a relatively greater cytologic action upon rapidly growing tumors than upon most tissues of adult organisms (Ludford (*23*)).

HEMORRHAGE-INDUCING PROPERTY OF COLCHICINE

Ludford (*8*) reported extensive necrosis and hemorrhage in tumors of mice which had received the highest tolerated doses of colchicine. Boyland and Boyland (*24*) drew attention to the similarity in the action of colchicine and a bacterial filtrate (*Bacillus typhosus*) in inducing hemorrhage in tumors. Both are more toxic to tumor-bearing mice than to controls. Both elicit a decrease in the ascorbic acid content of tumors and a fall in respiration and glycolysis. Neither induces a reduction in the glutathione content of tumors. In order to obtain these metabolic effects, "doses of colchicine approaching the toxic dose and ten times as great as those necessary to produce inhibition of mitosis are required" (Boyland and Boyland (*24*)). Andervont (*25*) found that colchicine and filtrate supplemented each other in producing hemorrhage and regression of transplantable mouse tumors, but their combined action was no more effective than a single injection of colchicine or filtrate. Previous work had demonstrated that the hemorrhage induced with bacterial filtrates was capable of bringing about complete regression of some spontaneous and transplantable mouse tumors (Duran-Reynals (*26*), Shwartzman (*27*), Fogg (*28*), and Andervont (*29*)). Recently Shear and Turner (*30*) isolated from culture filtrates of *Escherichia coli* and of *Serra-*

tia marcescens a polysaccharide fraction, which is extremely potent. The minimum dose of the polysaccharide obtained from *S. marcescens*, which produced detectable hemorrhage in approximately 50 percent of the tumors treated, was about 0.1 microgram. Shear (*31, p. 475*) stated that while its administration regularly produced hemorrhage and necrosis in primary tumors (induced with subcutaneous injections of 3,4-benzpyrene)—

> some portion of the neoplasm usually escaped destruction and continued to grow progressively until the death of the host. In the few instances in which the entire tumor appeared to have been severely affected, the animals developed symptoms of severe shock and died.

The general results of treatment with bacterial filtrates and colchicine are so similar that the possibility of their similar action in inhibiting tumor growth, namely, by vascular damage, must be considered. Identical views were expressed by Brues, Marble, and Jackson (*32*), who pointed out, as did also Andervont (*25*), that repeated injections of small doses of colchicine, sufficient to arrest mitosis but insufficient to induce hemorrhage, did not inhibit tumor growth.

A clue to the means whereby colchicine induces vascular damage is provided by the observations of Dustin and Chodkowski (*33*) on the action of the drug on newly formed capillaries in granulation tissue. The endothelial cells were particularly sensitive to mitotic poisoning. The number of arrested mitoses among endothelial cells rapidly declined with aging of the capillaries; few were detected after a week, and none after a month. Since concentrations of the drug in excess of those required to arrest mitosis in sensitive cells tend to be lethal to such cells, it is only to be expected that the capillary system of a rapidly growing tumor would be severely disrupted with high concentrations.

The hemorrhage induced with colchicine is not confined to tumors. It occurs also in the uterus of pregnant mice and results in abortion. With large doses of the alkaloid, the uterine hemorrhage is usually so severe as to be fatal.

PHYSIOLOGIC AND PHARMACOLOGIC ACTION

According to Dixon and Malden (*34*), colchicine exerts two distinct types of action. There is the immediate effect which is exerted on plain muscle and resembles the action of pilocarpine. "Peristalsis is increased, splenic, uterine and bronchiolar muscle show augmented tonus and movements and in every case the action is antagonized by atropine" (*34, p. 51*). However, Du Bilier and Warren (*35*) were unable to demonstrate that atropine influenced mitotic poisoning with colchicine when working with the Brown-Pearce rabbit carcinoma. The remote effect of colchicine poisoning is the gradual induction of paralysis of the central nervous system. The animal gradually passes into a state of narcosis in which the reflexes disappear, and which is followed by death from vasomotor and respiratory failure. The delayed toxic action was attributed to slow absorption into the central nervous tissues as is the case with bacterial toxins.

In any consideration of the possibility of transferring the results of treating animal tumors to the therapy of human tumors, it is pertinent to note Dixon and Malden's statement (*34, p. 64*):

> So far as our experiments go they suggest that the toxicity in different animals varies according to the degree of development of the cerebral hemispheres. That is to say, the dose of colchicine for any animal may be gauged by determining the relationship between the body weight and the weight of its cerebrum.

This implies the probability that the relatively greater sensitivity of the central nervous system of man to colchicine poisoning would mitigate against its use in concentrations comparable with those required to induce complete tumor regression in mice.

Colchicine, in common with certain other toxic substances including bacterial toxins, is relatively inactive when injected into hibernating mammals or frogs kept at a low temperature. Thus

frogs will survive in the cold injections of the drug, which are lethal when their temperature is raised to 30° C. (Führer (*36*)), and according to Hausmann (*37*) bats succumb to one-thirtieth of the dose that is tolerated by them when hibernating. To explain such results, it has been suggested that before colchicine is active, it must first be partially oxidized to form oxy-dicolchicine (Jacobj (*38*)), and that this does not occur in animals maintained at low temperatures; alternately, Lettré and Fernholz (*39*) attributed its relative inactivity in hibernating animals to cessation of cell division during hibernation. A more feasible explanation would be one which correlated lowered metabolism with reduced susceptibility to toxic agents.

Of considerable importance from the point of view of the chemotherapy of cancer is the problem to what extent is it possible to arrest mitosis without interfering with the metabolism of "resting" cells. This was investigated morphologically by examining, by dark-ground illumination, cells in tissue cultures which had been treated with colchicine in a concentration just sufficient to arrest mitosis (*8*). By this method the earliest changes in cellular degeneration are readily detectable since alterations in the mitochondria and granulation of the cytoplasm are indicative of the state of metabolic activity of cells (Ludford (*40*)). Morphologic study demonstrated that it was possible to arrest mitosis with colchicine without appreciable injury to resting cells. Confirmatory evidence was adduced by Brock, Druckrey, and Herken (*41*), who carried out a comparative investigation on the activity of a variety of compounds on the metabolism and arrest of mitosis of the fertilized sea urchin's egg. For each compound they determined the minimum concentration required to arrest mitosis and to increase the oxygen consumption by approximately 100 percent. Respiration was measured by the Warburg technique. Their results demonstrated that to arrest mitosis with colchicine it required only one-thousandth of the concentration necessary to induce the metabolic effect. With no other compound was there such a big difference. Contrariwise, some substances, e. g., veratrine, produced the metabolic effect in concentrations which failed to arrest mitosis.

II. ACTION OF COLCHICINE ON TUMOR GROWTH

TREATMENT OF ANIMAL TUMORS WITH COLCHICINE

Amoroso (*4*) was the first to report inhibition of tumor growth with colchicine. In a preliminary note he referred to experiments carried out with mouse carcinoma 63 in which he obtained complete regression of malignant growth. He also treated a Sealyham dog which had an ulcerated carcinoma of the buccal mucous membrane. A biopsy was made which confirmed the clinical diagnosis. Injections of colchicine were given over a period of approximately 6 weeks, at the end of which "only a small scar remained at the site of the original growth." Amoroso has not published a more detailed account of his work.

Williamson (*42*) treated a 7-year-old mare with tumors on the head, trunk, and right forelimb which were diagnosed histologically as spindle-cell sarcoma. Injections of the alkaloid were given intramuscularly on alternate days over a period of 40 days, the dose varying from 0.5 to 1.0 cc. of "a solution containing one milligramme of colchicine per c. c." (*42, p. 24*), and on the forty-sixth day a last dose of 10 cc. was given, which produced an intense reaction. Presumably the tumors regressed completely, since the horse was reported to have regained her normal health.

Peyron, Lafay, and Kobozieff (*43*) and Peyron, Poumeau-Delille, and Lafay (*44, 45*) carried out investigations on the action of colchicine on the Shope rabbit papilloma. They were able to bring about complete regression of the papillomatous growths with eight or nine injections, averaging 1.5 mg., distributed over a period of 3 to 4 weeks. Cured rabbits were immune to the virus.

Very striking results were obtained by Lits, Kirschbaum, and Strong (*46*) in the treatment of a malignant lymphoid tumor of the C3H strain of mice. This tumor was "transplanted into 104 mice with no failures (13 transfer generations) and no spontaneous regressions" (*46, p. 199*). Following the injection of one-fortieth milligram of colchicine into mice starting on the fourteenth day after transplantation, and repeating the treatment on the seventeenth and twentieth days, they obtained complete regression of some tumors. But "in every instance there was recurrence of treated tumors, even if growth was suppressed for as long as five weeks" (*46, p. 212*). In spite of repeated colchicine injections, death from leukemia always ensued. The marked response of this tumor to treatment was attributed to the extreme sensitivity of malignant lymphocytes to the alkaloid.

The significant observation was made by Boyland and Boyland (*47*) that different tumors varied in their response to treatment. Thus a sarcoma of the dilute brown strain of mice was more sensitive than the Crocker sarcoma in mixed stock mice. Of the various types of tumors investigated, spontaneous mammary carcinomas exhibited the least reaction, chemically induced sarcomas in mixed stock mice rather more, and the most sensitive were two strains of grafted tumors (one a carcinoma, the other a sarcoma) in pure-line mice. The Boylands attempted to influence the action of colchicine by preliminary treatment with ascorbic acid and also with 2-methyl-naphthoquinone, which has high vitamin K activity, but the results were negative.

Guyer and Claus (*48*) injected rats bearing the Flexner-Jobling carcinoma with 0.1 mg. colchicine per 100 gm. body weight. Fifteen hours later, distilled water was injected directly into each tumor "in as great quantity as it would hold" (*48, p. 273*). In 59 of 103 rats (57 percent) so treated, the tumors completely regressed. Some healed entirely after 1 treatment. Others necessitated repetition of the treatment. Injections of distilled water alone into 47 other rats resulted in approximately the same percentage of regressions as in untreated controls (3–4 percent).

There is general agreement that repeated injections of small doses of colchicine fail to influence the rate of growth of mouse tumors (Poulsson (*49*), Ludford (*8*), Clearkin (*50*), and Brues, Marble, and Jackson (*32*)), unless treatment is commenced within a day or two of transplantation, when growth is somewhat retarded (Lettré (*51*)).

The writer has carried out a series of experiments on the treatment of 17 different strains of transplanted tumors in mice of inbred strains and in hybrids, and with 49 spontaneous mammary carcinomas. Detailed results will be published separately with evidence to substantiate the following conclusions:

(1) That with injections of colchicine it is possible to bring about complete regression of some tumors, but only by employing large doses of the drug just below the minimum lethal dose, and considerably in excess of those which arrest mitosis.

(2) The tumors which respond most to treatment are the soft, highly cellular, rapidly growing type. Very slow growing tumors are resistant. The degree of response is, in general, proportional to the rate of tumor growth in any one inbred strain of mice.

(3) Histologic structure, in as far as it determines the consistency of a tumor, influences its sensitivity to the drug. Thus a fibrosarcoma responded less to treatment than did a spindle-cell sarcoma which grew at approximately the same rate.

(4) Partially inactivated colchicine or a less toxic derivative (amine) possesses no advantage since proportionally larger doses are required to obtain the same clinical result.

(5) The various inbred strains of mice differ with respect to the dose tolerated, and also in the sensitivity of their newly formed capillaries to the toxic action of the drug.

(6) When the same tumor is grown in different strains of mice, the extent of the hemorrhage induced with the same dose of colchicine varies. The greatest regression of tumor growth occurs in those mice most sensitive to the hemorrhage-inducing action of the drug.

It is therefore concluded, in confirmation of previous tests, that regression of tumor growth subsequent to colchicine treatment is primarily the result of hemorrhage following the disruption of newly formed capillaries.

COMBINED COLCHICINE AND IRRADIATION TREATMENT OF ANIMAL TUMORS

The results of combined treatment which have been published have failed to sustain the hopes that were raised by Amoroso's original observations that colchicine might considerably increase the radiosensitivity of tumors. DuBulier and Warren (*35*), who worked with the Brown-Pearce rabbit epithelioma, pointed out one practical difficulty: "Tumors of the same age and from the same generation but in different sites, even in the same animal, do not give consistent results when treated with the same dosage of colchicine" (*35, p. 968*). Hence there is the difficulty of deciding exactly when to apply irradiation unless biopsies are first made. However, those who have employed the combined technique have usually circumvented this difficulty by first ascertaining the average time after injection of the drug when the maximum arrest of mitosis occurs in a group of animals.

That irradiation can under certain conditions increase the growth-inhibitory action of colchicine was demonstrated by Guyer and Claus (*52*). They carried out two types of experiments. Rats with the Flexner-Jobling carcinoma were given 0.2 mg. of colchicine per 100 gm. body weight. Fifteen to eighteen hours later, thin slices were cut from the tumors of these rats and from those of controls. The tumor slices were exposed in the same dish to X-rays (1,500 r dosage) and then grafted into other rats. Slices of tumors from rats that had received only the colchicine treatment and from the untreated controls were also grafted. From the results of 3 experiments in which a total of 201 rats was employed, the following percentages of successful takes were calculated:

	Percent
Controls	85
Colchicine plus X-rays	20
X-rays only	84.5
Colchicine only	78

When the combined treatment was applied to rats transplanted with the same tumor, colchicine was less effective in enhancing the action of irradiation. Sixteen days after transplantation, 91 tumor-bearing rats were divided into 3 groups. Thirty-two received 0.1 mg. colchicine per 100 gm. body weight and 15 hours later were irradiated (188 r); treatment was applied twice weekly. Twenty-nine rats received X-ray treatment only, and thirty colchicine only. The tumors subjected to the combined treatment grew "notably less on the average than those of either of the other groups" (*52, p. 568*). The results at the end of 6 weeks, calculated as percentages, are as follows: Of the animals receiving colchicine plus X-rays, 47 percent were completely healed and 12.5 percent were dead; of those receiving X-rays alone, 38 percent were healed and 10 percent were dead; and of the rats receiving colchicine alone, 27 percent were healed and 33 percent were dead. Regressions in controls were given in a later paper (Guyer and Claus (*48*)) as between 3 and 4 percent.

Brues, Marble, and Jackson (*32*) pointed out that the doses of the alkaloid used in the preceding experiments "are close to the lethal, and are in the dosage range producing metabolic as well as mitotic changes." The results are therefore scarcely relevant to the problem whether colchicine, by arresting the mitosis of large numbers of tumor cells, would thereby increase their radiosensitivity. So Brues and his collaborators gave maximal sublethal daily doses of the drug to mice with sarcoma 180 and to rats bearing the Walker rat sarcoma 256. Thus only mitotic arrest was effectuated. Irradiation was carried out daily 16 hours after colchicine injections, beginning the eleventh and ending the twentieth day after transplantation. The daily dose averaged 300 r. Both mice and rats were divided into four groups, which received (1) colchicine plus X-rays, (2) colchicine alone, (3) X-rays alone, and (4) no treatment. The results showed that colchicine failed to influence the action of irradiation treatment. Further, it was found that by employing the same doses of colchicine it was not possible to pre-

vent recurrence or metastases after the surgical excision of tumors, as could be done with irradiation.

An investigation planned on similar lines was carried out by Hirshfeld, Tennant, and Oughterson (*53*), employing a mouse mammary carcinoma (Yale 1). With colchicine alone they obtained retardation of tumor growth and prolongation of life. The injection of colchicine (0.001 mg. per gram body weight) followed 8 to 10 hours later by a single dose of X-rays (2,500 r) gave a slightly higher percentage of cures than did irradiation alone, but when the dose of X-rays was doubled (5,000 r), colchicine did not appreciably influence the 48-percent cures obtained with irradiation alone. It could not, therefore, be claimed that the results of the combined treatment that have been published to date have been particularly encouraging.

TREATMENT OF HUMAN TUMORS

According to Lits (*2*), the first empirical observations concerning the action of colchicine on tumors were recorded by Dominici (*54*). He reported improvement in the general condition of cancer patients treated with the drug. Oughterson, Tennant, and Hirshfeld (*55*) studied its cytologic effects on 21 patients. Fifteen of these had control biopsies, then 9½ hours later either a second biopsy was made or the entire tumor was removed. Eleven showed definite arrest of mitosis at metaphase, which could be induced with 1.5 mg. of the drug. A case of anaplastic carcinoma of the sigmoid colon was subjected to most detailed study.

> The tumor was delivered on the abdominal wall according to the Mikulicz technique with its blood supply intact and was readily accessible for biopsy (*55, p. 663*).

Pieces of the tumor were first removed as control, then 4.0 mg. of colchicine was injected subcutaneously. Biopsies were made 5 and 9½ hours after the injection, and after 12 hours the tumor was excised. A gradual increase in arrested mitoses was observed up to the twelfth hour, when there were more than 7 times as many mitotic figures as in the control. The doses of colchicine employed in this work did not result in any general toxic symptoms, but there was local irritation and "the discomfort in some patients was of such a degree as to make it desirable to use some other method of administering the drug" (*55, p. 664*). The suggestion was made that the colchicine technique might be of value for obtaining a more accurate index of the rate of growth of the tumor than can be obtained by ordinary methods.

Brücke and Hueber (*56*) combined local injections of small doses of the alkaloid with irradiation in the treatment of a subcutaneous metastasis about the size of a hazelnut, in a patient with gastric carcinoma. Daily for 8 days 0.1 mg. of colchicine was injected in the neighborhood of this metastasis. Then, commencing on the fourth day, irradiation (200 r) was carried out daily 1½ hours after the injection and was continued alone on 3 subsequent days. Another similar metastasis received only the irradiation treatment. Since the patient died 11 days later, a histologic examination was made. The metastasis that had received the combined treatment was replaced by necrotic tissue, while the other was said to be unaffected. The same authors refer to another patient, with an axillary-gland metastasis, to which they applied the same combined treatment and noted a decrease in edema of the arm and the curing of paresthesia in the hand.

Of special interest is the report of Seed, Slaughter, and Limarzi (*57*) recording the treatment of four advanced cases of carcinoma, since the results were so similar to those obtained in experiments with laboratory animals. Two of the patients died of colchicine poisoning a few days after commencing treatment. In both cases, there were fever, acute diarrhea, severe agranulocytic leukopenia, anemia, and a tendency to excessive bleeding, "evidence of suppression of all the blood-forming elements." One of these two patients, a 65-year-old female with a scirrhous-duct carcinoma, received intramuscularly 12 mg. of the drug in the course of

4 days, and the other, a 44-year-old male with carcinoma of the rectum, received 22 mg. spread over 5 days.

A third case was that of a 57-year-old woman with adenomedullary carcinoma of the breast, which was continuous with a large mass of fused metastatic glands. The following is a summary of the case history of this patient:

First day, 1 mg. colchicine was given intramuscularly; second day, 2 mg. was given; fourth day, 4 mg., and fifth day, 7 mg. On the fifth day, her temperature was 101° F., her pulse 120, and she was nauseated and vomited several times. On the sixth day, she received 1,000 cc. dextrose solution intravenously; on the tenth day, the patient was nauseated; there was marked prostration; she was given 3,000 cc. fluid intravenously, and 500 cc. of citrated blood. By the twelfth day, the surface of the tumor had changed to a dull gray color; on the sixteenth day, she was given 300 r. over the breast; on the eighteenth, the surface of the tumor was observed to be necrotic; on the nineteenth day 350 r. were given to the right supraclavicular region. Two days later, the hair on her scalp fell out, the tumor in the breast and that in the axilla began to shrink rapidly, and there was cessation of pain. Two days later, she was given 2 mg. colchicine. By the thirty-first day, the entire tumor mass had shrunk to a third of its original size. On the thirty-third day, she was given 2 mg. of colchicine. By the fiftieth day, when she received 4 mg. of the drug, the ulcerating area was contracting and acquiring an epithelial covering. Two milligrams of colchicine was given on the fifty-fourth day; on the fifty-ninth day, it was noted that the tumor was increasing in size. From the sixtieth through the sixty-fifth day, 1 mg. of the drug was given daily. On the seventy-seventh day, the tumor was growing rapidly, and by the ninety-ninth day it was growing at a much accelerated rate.

The fourth patient treated was a 54-year-old man with anaplastic carcinoma on the left side of the neck. He had previously received extensive high voltage roentgen therapy. The tumor had regressed, but there was recurrence of the growth which was now radioresistant. The following is a summary of the colchicine treatment and its results:

The first day, 2 mg. of colchicine, the second day 4 mg., and the third day also 4 mg. were administered. Toxic effects were noted after 10 mg. was given. On the seventh day, the skin over the surface of the tumor was cyanotic. On the eleventh day, also on the fifteenth, he received 2 mg. of colchicine and again on the twenty-fourth day, when there was superficial necrosis. Two days later, 3 mg. colchicine was given. On the twenty-seventh day, a definite slough appeared; by the thirtieth day, when 4 mg. was given, necrosis was extensive. Almost the entire tumor could be scooped out. It consisted of a cheesy mass of necrotic tissue without blood supply or sensation, but there was a thin peripheral shell of live tumor tissue. The patient was ill and had a fever. On the fortieth day 2 mg., and on the forty-fifth 3 mg. of colchicine were given. On the forty-sixth day, the center of the tumor continued to slough out, but the periphery began to extend with startling rapidity. On the fifty-fourth day, the patient died of cachexia. The tumor in the chest wall consisted of a cystic mass with relatively thin carcinomatous wall and necrotic, liquefied center.

It is significant that the opinion is expressed in both of these cases for which the history is given, that the tumors, after the primary regression, began to grow at an accelerated rate.

Seed, Slaughter, and Limarzi stated that in selecting their cases for treatment they were influenced by the present writer's observations (*8*) that rapidly growing tumors are most sensitive to mitotic poisoning. The treatment which they adopted would appear to have been directed to obtaining the maximum intensity and prolongation of mitotic poisoning. Evidence is adduced in preceding paragraphs that the regression of animal tumors induced with colchicine is primarily the result of vascular damage. The writer's experiments indicate that the greatest injury to the blood vessels of tumors is effected by administering the maximum nonlethal dosage of the drug, either in a single dose or in several doses over a period of a few hours, then allowing the animal to recover sufficiently before repeating the injections. By applying this technique to mice with spontaneous mammary tumors, there was obtained considerable arrest of tumor growth, but only 2 of 49 tumors completely regressed. It is possible that the application of the same method of administering the drug to human patients with rapidly growing tumors might have given better results. The conclusions of Seed, Slaughter, and Limarzi, however, demand serious consideration in any attempts to employ

colchicine in the treatment of human patients. They wrote:

> Although the rapidly growing cancer cells are much more susceptible to the poison, the concomitant general toxic effect is much too great to expect any curative effect.

GENERAL CONCLUSIONS

Dustin in his papers on "poisons caryoclasiques" frequently commented on the similarity of their cytologic action to that of irradiation, and he described the effect of colchicine as being characterized by "un véritable radiomimétisme" (10). He pointed out that both mitotic poisons and irradiation exert their action specially "sur les chromatines nucléaires." Both are specially injurious to dividing cells. Also, certain type of cells, such as those of the vortex of the thymus, which exhibit special sensitivity to all mitotic poisons are also highly radiosensitive. Reference was made previously to the almost complete regression of a transplantable lymphoid tumor (Lits, Kirschbaum, and Strong (*46*)), which was induced with colchicine treatment but which always recurred. Lymphosarcomas exhibit a similar high degree of sensitivity to irradiation. Likewise, they also invariably recur (Warren *58*)). Again, the tolerance which tumors acquire after prolonged colchicine treatment is at least analogous to the radioresistance of tumors that previously respond to irradiation.

Concerning the nature of the injury to the mitotic process brought about with colchicine and irradiation, it is necessary to emphasize certain fundamental differences. As previously described, colchicine is a "spindle poison," but it is also able to bring about contraction of chromosomes and even their fusion. The mitotic effects of irradiation are less specific. The wave of mitosis which supervenes upon the inhibitory phase is characterized by such abnormalities as fragmentation of chromosomes and irregularities in their separation at anaphase, as well as by multipolar figures. Nevertheless, as in the case of colchicine, the mitotic effects of irradiation are greatest on the fastest growing tumors (Ludford (*59*)).

There is yet a further similarity in the action of colchicine and irradiation. In addition to the mitotic effects, they both induce vascular damage. This may be less important in radiotherapy than in colchicine treatment. Since the cytologic action of the drug is nonspecific and therefore operative on all dividing cells of the organism, it is impossible to administer doses lethal to all the cells of a tumor without destroying sensitive tissues in other parts of the body which are necessary for the maintenance of life (e. g., blood-forming tissues). Radiotherapy being essentially a local treatment is obviously not subject to such limitations of dosage. Thus it is the high degree of sensitivity of the endothelium of the newly formed tumor capillaries to colchicine poisoning, and the wide repercussions of vascular damage on tumor growth, which become of predominant importance in treatment with the alkaloid.

The outstanding difficulties encountered in the colchicine treatment of tumors are the result of the lack of cytologic specificity in its action, and the concomitant general toxicity (especially to the central nervous system) of the dosage necessary to induce regression of malignant growth. Search for a potent antidote to colchicine poisoning that would rectify the effects of overdosage has so far been unsuccessful. Derivatives of the alkaloid have been prepared in the hope that some modification in chemical constitution might result in a compound towards which malignant cells would exhibit differential susceptibility. Nothing of that kind has yet been found. In general, the greater the alteration in chemical structure the less active is the derivative. Modifications of the methoxymethylene group (on ring C) are illuminating in this respect. By designating the activity of colchicine on the basis of its activity on cells in vitro as unity, the potencies of some derivatives have been calculated from the results of Lettré and the writer and are given in table 1. Derivatives of this type, which are less toxic and correspondingly less potent, possess no ad-

vantage over colchicine itself in the treatment of tumors since proportionately larger doses are required to obtain similar clinical results.

TABLE 1.—*Calculated potencies of colchicine and some of its derivatives*

Compound	Partial formula	Activity
Colchicine	R=CH-OCH_3	1
Colchiceine	R=CH-OH	1/500
Colchiceineamine	R=CH-NH_2	1/10
Ethylcolchiceine	R=CH-OCH_2-CH_3	1/8
Propylcolchiceine	R=CH-OCH_2-CH_2-CH_3	1/80
Butylcolchiceine	R=CH-OCH_2-CH_2-CH_2-CH_3	1/250

The chemical constitution of colchicine is still the subject of investigation, and Cook and his colleagues (*60*) have adduced evidence that the structural formula of Windaus can no longer be considered valid. Extension of our knowledge of the chemical constitution of colchicine and related substances may reasonably be expected to lead to the discovery of new physiologically active compounds. Further progress is dependent upon finding among them some proclivity to a specific inhibition of malignant growth.

SUMMARY

Colchicine arrests cell division by preventing the formation of the mitotic spindle. It is impossible to inhibit mitosis of malignant cells without simultaneously effecting the same action on other dividing cells of the organism.

Colchicine causes vascular damage to rapidly growing tumors, since endothelial cells of newly formed capillaries are particularly sensitive to mitotic poisoning.

Regression of tumor growth followed by recurrence has been reported after treatment of the drug in domestic and laboratory animals and in man.

Complete regression of some animal tumors has been induced by employing doses of the drug far in excess of those required to arrest mitosis, and just short of the minimum lethal dose.

Evidence has been adduced that when regression of tumor growth occurs, it is primarily the result of vascular damage.

Less toxic derivatives of colchicine that have been investigated have not possessed any advantage since proportionately larger doses were required to produce similar clinical results. In general, the greater the alteration in chemical structure, the less active is a derivative in inducing tumor regression.

Preliminary treatment with colchicine has not been found to increase significantly the radiosensitivity of tumors.

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CANCER MORTALITY AND MARITAL STATUS; AN ANALYSIS OF DEATHS ATTRIBUTED TO CANCER AMONG THE WHITE POPULATION OF NEW YORK CITY DURING 1939–41 [1]

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Some years ago, one of us joined in the publication of data on the recorded mortality from cancer in New York City (*1*). A study of cancer mortality by marital status, begun at that time, was not completed because we were not satisfied that the population estimates then available were sufficiently accurate with respect to the marital status of the population in the years for which the reported cancer mortality could be classified by color, sex, age, and site. However, data from the 1940 census (*2*) have since made such a study possible.

Our interest in cancer mortality by marital status arose from our own observation and the reports of several others that the death rate from cancer of the breast was higher among women whose marital status was reported on death certificates as single than that among women reported at the time of death to have been married, widowed, or divorced. (Hereafter, these last-mentioned persons will be grouped under the terms "married" or "other.") The findings presented herein are based upon the recorded mortality from cancer among the white population of New York City, 15 years of age or older, during the 3-year period 1939–41. They include a total of 34,019 deaths, distributed as follows: Single males, 2,294; single females, 2,255; other males, 15,011; and other females, 14,459 (table 1). Of the 221 additional deaths charged to cancer during the 3 years but not included in this paper, all at ages under 15 years, 125 were single males, and 96 were single females.

Most statements of cause of death reported on death certificates are not based upon necropsy; and when cancer is the true cause of death, that fact is not always reported. Furthermore, when death is ascribed to cancer, the site of a metastatic growth is much too frequently reported instead of the organ primarily affected. On the other hand, it is known that among persons in the "cured cancer" group, deaths which resulted from other causes have, nevertheless, been charged to cancer by the certifying physician. However, because of the high degree of skill of our medical practitioners, the high proportion of our sick who are hospitalized, and the relatively high percentage of necropsies which help to form the basis of our data, there is reason to believe that causes of death are reported more accurately for New York City than for any other population of the same size in the United States. The fact that our data, drawn from a comparatively large population, reveal some differences in the pattern of cancer mortality between single persons and others would seem to justify their publication.

CANCER, ALL FORMS, ALL SITES

During the years 1939–41, single persons as a group exhibited slightly higher mortality rates for cancer, all forms, all sites, than did those who had been married, the excess among males being 3 percent and among females 8 percent.[3] As can be seen in figure 1, however, the age-specific death rates were not consistently lower for those who had married. For both sexes, the married had higher rates under 35, but lower rates from 35 to 64. After 65 years of age, the rate among bachelors was 10 percent lower than among other males. In contrast with this finding, the cancer death rate among spinsters in the age group 65 to 74 was the same as that for other

[1] A revised version, with 1941 data included, of a paper read by Thomas J. Duffield before the cancer symposium held in conjunction with the seventy-second annual meeting of the American Public Health Association in New York City, October 11, 1943.

[2] At present commissioned as Assistant Sanitarian in the reserve of the U. S. Public Health Service.

[3] In this and in similar statements referring to more than one age group, effects of age differences have been eliminated by use of the standard million, England and Wales, 1901. (See tables 2 and 3.)

TABLE 1.—*Population reported in 1940 census [1] and number of deaths from cancer by site, according to age, sex, and marital status, white persons, city of New York, 1939–41*

WHITE MALES

Site	Age group															
	Total, 15 and over		15–24		25–34		35–44		45–54		55–64		65–74		75+	
	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other
All sites	2,294	15,011	116	10	93	177	214	785	519	2,778	666	4,682	486	4,486	200	2,093
Buccal cavity and pharynx	185	766	7	.....	4	5	12	29	42	130	57	257	48	250	15	95
Stomach	378	2,858	1	.....	10	25	37	146	80	485	115	914	106	877	29	411
Digestive tract [2] and peritoneum	707	5,400	13	2	25	49	57	247	148	886	230	1,689	164	1,734	70	793
Respiratory system	402	2,493	10	2	11	18	55	146	129	659	131	872	52	613	14	183
Genito-urinary organs	321	2,259	25	2	16	24	17	72	61	280	78	598	70	784	54	499
All others	301	1,235	60	4	27	56	36	145	59	338	55	352	46	228	18	112
Population	995,061	1,766,166	536,330	37,307	237,534	371,884	105,993	493,407	62,117	435,835	34,006	264,641	15,021	124,502	4,060	38,630

WHITE FEMALES

All sites	2,255	14,459	71	20	103	314	246	1,382	454	3,027	559	3,982	521	3,704	301	2,030
Buccal cavity and pharynx	27	133	3	1	3	2	2	10	2	36	9	32	6	30	2	22
Stomach	219	1,919	4	.....	8	17	11	106	26	286	58	521	61	628	51	361
Digestive tract [2] and peritoneum	634	4,656	11	4	19	48	47	276	113	774	153	1,349	184	1,423	107	782
Respiratory system	87	625	5	.....	5	13	7	43	17	114	29	195	17	175	7	85
Uterus	210	1,846	3	1	9	73	25	255	41	486	65	508	53	369	14	154
Other genital organs	199	951	9	4	12	28	28	142	54	293	54	258	29	166	13	60
Breast	617	2,831	4	3	25	77	102	414	162	732	150	704	109	564	65	337
All others	262	1,498	32	7	22	56	24	136	39	306	41	415	62	349	42	229
Population	869,502	1,982,629	490,607	114,352	184,456	488,443	83,130	515,807	51,019	413,626	33,433	257,946	19,902	141,614	6,955	50,841

[1] Population, fourth series, 1940, Bureau of the Census, 1943, p. 26.
[2] Other than stomach.

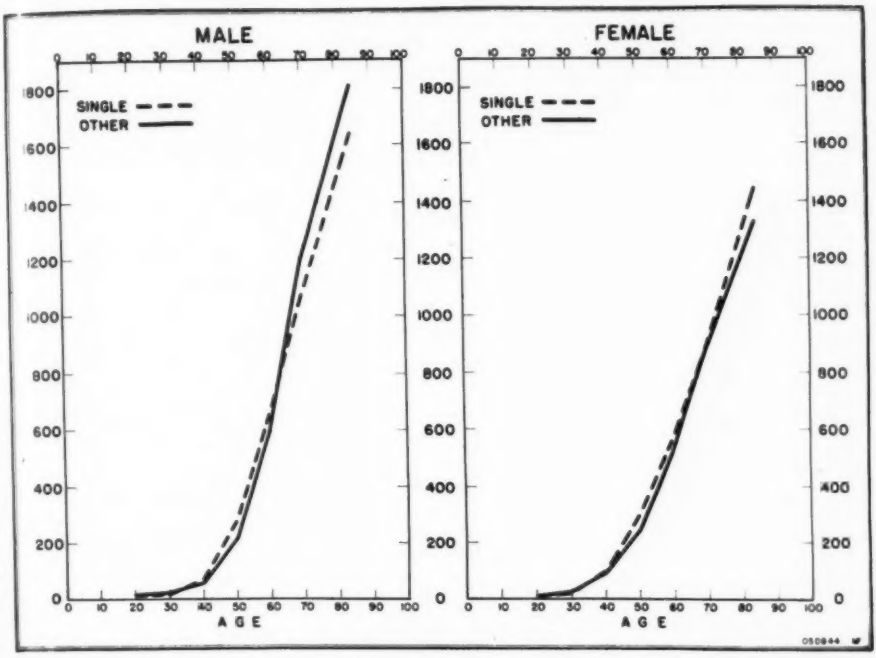

FIGURE 1.—Cancer, all sites, by sex and age according to marital status, death rates per 100,000 white population, city of New York, 1939–41.

women, but at ages over 75 years it was almost 10 percent higher than for those who had been married.

CANCER OF THE BREAST, FEMALES

In our experience, the death rate from breast cancer among single women at all ages over 15 years was more than 1½ times that among those who had married. At 15 to 34 years of age, when the mortality is comparatively low, the rate among the married was about one-third higher than that among the single,[4] (3), but at all ages over 34 years, single women had much higher death rates from breast cancer (fig. 2). After the normal child-bearing period, that is at ages 45 and over, the mortality among single women averaged 58 percent higher, the greatest difference being at ages 45 to 54, when the rate for spinsters was 79 percent greater than the rate for others of their sex.

We do not pretend to know why this is so. Can it be due to the fact that the married woman is under more regular medical supervision, especially during the child-bearing period, and if a cancer develops, it is frequently detected early enough to effect a "cure"? Or may it be due to the fact that a single woman leads an abnormal life, characterized by endocrine disturbances? In any case, our data confirm observations previously reported by the Registrar-General for England and Wales (*3*) and by Dorn (*4*) for Australia. Thus, they lend support to the theory that the bearing and rearing of children decrease the likelihood among women of death from cancer of the breast.

[4] While the rates were not significantly different among the married and the single under 35 years of age, they were consistent with the findings from unpublished data for New York City for 1937 and with those for England and Wales for 1911–20 and 1930–32.

TABLE 2.—*Cancer mortality by site, according to age, sex, and marital status, white persons, city of New York, 1939–41, rates per 100,000 population*

WHITE MALES

Site	Age groups															
	Total, 15 and over [1]		15–24		25–34		35–44		45–54		55–64		65–74		75+	
	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other	Single	Other
All sites	197.7	191.3	7.2	8.9	13.1	15.9	67.3	53.0	278.5	212.5	652.8	589.7	1,078.5	1,201.1	1,642.0	1,806.0
Buccal cavity and pharynx	16.6	9.5	.4	0	.6	.4	3.8	2.0	22.5	9.9	55.9	32.4	106.5	66.9	123.2	82.0
Stomach	34.4	36.0	.1	0	.4	2.2	11.6	9.9	42.9	37.1	112.7	115.1	235.2	234.8	238.1	354.6
Digestive tract [2] and peritoneum	64.1	68.8	.8	1.8	3.5	4.4	17.9	16.7	79.4	67.8	225.5	212.7	363.9	464.2	574.7	684.3
Respiratory system	32.1	30.3	.6	1.8	1.5	1.6	17.3	9.9	69.2	50.4	128.4	109.8	115.4	164.1	114.9	157.9
Genito-urinary organs	29.6	30.3	1.6	1.8	2.2	2.2	5.3	4.9	32.7	21.4	76.5	75.3	155.3	209.9	443.3	430.6
All others	21.0	16.3	3.7	3.6	3.8	5.0	11.3	9.8	31.7	25.9	53.9	44.3	102.1	61.0	147.8	96.6

WHITE FEMALES

All sites	183.9	170.1	4.8	5.8	18.6	21.4	98.6	89.3	296.6	243.9	557.3	514.6	872.6	871.9	1,442.6	1,330.9
Buccal cavity and pharynx	2.0	1.6	.2	.3	.5	.1	.8	.6	1.3	2.9	9.0	4.1	10.0	7.1	9.6	14.4
Stomach	18.5	22.5	.3	0	1.4	1.2	4.4	6.9	17.0	23.0	57.8	67.3	102.2	147.8	244.4	236.7
Digestive tract [2] and peritoneum	53.0	54.7	.7	1.2	3.4	3.3	18.8	17.8	73.8	62.4	152.5	174.3	308.2	334.9	512.8	512.7
Respiratory system	6.9	7.3	.3	0	.9	.9	2.8	2.8	11.1	9.2	28.9	25.2	28.5	41.2	33.5	55.7
Uterus	17.2	21.5	.2	.3	1.6	5.0	10.0	16.5	26.8	39.2	64.8	65.6	88.8	86.9	67.1	101.0
Other genital organs	15.8	11.2	.6	1.2	2.2	1.9	11.2	9.2	35.3	23.6	53.8	33.3	48.6	39.1	62.3	39.3
Breast	51.0	33.2	.3	.9	4.5	5.3	40.9	26.8	105.8	59.0	149.6	91.0	182.6	132.8	311.5	221.0
All others	19.4	18.1	2.2	2.0	4.0	3.8	9.6	8.8	25.5	24.7	40.9	53.6	103.8	82.1	261.3	150.1

[1] Age standardized, England and Wales, 1901.
[2] Other than stomach.

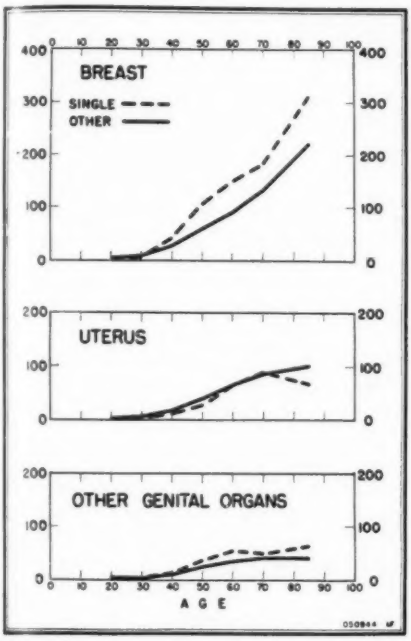

FIGURE 2.—Cancer of the breast, uterus, and other genital organs, by age according to marital status, death rates per 100,000, white females, city of New York, 1939–41.

CANCER OF THE UTERUS

If the mortality from cancer of the breast is higher among spinsters, what may we expect with regard to the mortality from cancer of the uterus? Is there any difference between the behavior of cancer of the cervix and cancer of the corpus? Unfortunately, even in a city like New York, too many physicians are still vague when reporting deaths from uterine cancer and do not differentiate between cancers of the cervix and those of the body of the uterus. We are not in a position, therefore, which would permit us to make any definite report on the latter question.

However, since most uterine cancers arise in the cervix, presumably on the sites of lacerations suffered at childbirth, it has long been held that married women have a great excess of mortality from cancer of the uterus. The findings of the first investigation of the kind to lend statistical support to this generally accepted view were published in the seventy-sixth annual report of the Registrar-General of Births, Deaths, and Marriages in England and Wales (London, 1915) and included observa-

TABLE 3.—*Cancer mortality by site according to age and sex, white persons, city of New York, 1932–41, ratio of rates among single persons to others*

Site	Age group							
	Total, 15 and over [1]	15–24	25–34	35–44	45–54	55–64	65–74	75+
WHITE MALES								
All sites	1.03	0.81	0.82	1.27	1.31	1.11	0.90	0.91
Buccal cavity and pharynx	1.73		1.25	1.90	2.27	1.73	1.59	1.50
Stomach	.95		.64	1.17	1.16	.98	1.00	.67
Digestive tract [2] and peritoneum	.93	.45	.80	1.07	1.17	1.06	.78	.84
Respiratory system	1.06	.35	.94	1.75	1.37	1.17	.70	.73
Genito-urinary organs	.97	.89	1.00	1.08	1.53	1.02	.74	1.03
All others	1.29	1.03	.76	1.15	1.22	1.22	1.67	1.53
WHITE FEMALES								
All sites	1.08	0.83	0.87	1.10	1.22	1.08	1.00	1.08
Buccal cavity and pharynx	1.24	.70	3.65	1.24	.45	2.20	1.41	.67
Stomach	.82		1.17	.64	.74	.86	.69	1.03
Digestive tract [2] and peritoneum	.97	.64	1.03	1.06	1.18	.87	.92	1.00
Respiratory system	.94		1.02	1.01	1.21	1.15	.69	.60
Uterus	.80	.70	.32	.61	.68	.99	1.02	.66
Other genital organs	1.41	.52	1.16	1.22	1.50	1.62	1.24	1.59
Breast	1.54	.31	.85	1.53	1.79	1.64	1.38	1.41
All others	1.07	1.10	1.05	1.09	1.03	.76	1.26	1.34

[1] Rates are age standardized, England and Wales, 1901.
[2] Other than stomach.

tions on the relative frequency of cancer of the breast and of the generative organs among the single and the married during 1911–13. Data for subsequent years, published by the Registrar-General, continue to show a large excess of mortality from uterine cancer among the married and the widowed.

These findings are confirmed by the data for New York City for the period 1939–41, when the mortality from cancer of the uterus was 25 percent higher among women who had married than among those reported as single. The rates for spinsters were lower at all ages except 55 to 74, at which age period they were apparently equal to the rates for women who had married.

CANCER OF OTHER FEMALE GENITAL ORGANS

For the other female genital organs, we find that the cancer death rate for single women is higher than that for married women. This finding is contrary to the one for mortality from uterine cancer but similar to that noted for cancer of the breast. However, the excess of mortality from cancer of the genital organs other than the uterus among single women for all ages 15 years and over is only 41 percent as compared with an excess of 54 percent for cancer of the breast. Similar data have been reported from England and Wales.

Figure 2 shows that among spinsters the death rate from cancer of the uterus peaked sharply at ages 65 to 74 in contrast with a decrease at those ages recorded for cancer of other genital organs. In view of the fact that variations of as few as 10 deaths could eliminate either fluctuation, this observation is of doubtful significance and hence may have no real meaning.

CANCER OF THE GENITO-URINARY ORGANS IN THE MALE

The differences in the mortality from cancer of the genito-urinary organs between the single and other males (fig. 3) are also of interest. For all ages 15 years and over, combined, no real difference was found between the rates for the single and others. However, the mortality rate among single men was 50 percent higher than for the married at ages 45 to 54, but 25 percent lower at ages 65 to 74.

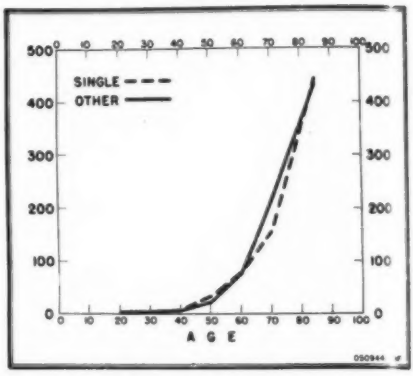

FIGURE 3.—Cancer of the genito-urinary organs, by age according to marital status, death rates per 100,000, white males, city of New York, 1939–41.

Married men have a less favorable mortality from cancer of the prostate, particularly at ages 55 to 74. While their mortality from cancer of the other genito-urinary organs was higher after 65 years of age, the excess was not sufficient to give them a less favorable lifetime experience. The rates are shown in table 4.

TABLE 4.—*Cancer of genito-urinary organs in white males, according to age and marital status, per 100,000, city of New York, 1939–41*

Site	Marital status	Age group					
		15 and over	35–44	45–54	55–64	65–74	75+
Prostate	Single	13.7	0.3	5.2	23.5	95.1	303.8
	Other	14.7	.2	3.7	33.5	120.5	263.2
Other genito-urinary organs	Single	15.9	5.0	26.8	53.0	59.9	139.5
	Other	15.6	4.7	17.7	41.8	89.4	197.4

CANCER OF THE BUCCAL CAVITY AND PHARYNX

The buccal cavity in males is the only site other than those mentioned previously in which single and married per-

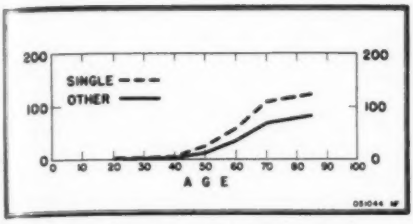

FIGURE 4.—Cancer of the buccal cavity and pharynx, by age according to marital status, death rates per 100,000, white males, city of New York, 1939–41.

sons had markedly different rates of cancer mortality. At all ages 15 years and over, combined, the mortality among single men exceeded that among married men by 73 percent. For the 3 years studied, the rate for bachelors far exceeded that for men who had married, throughout the life span (fig. 4).

Much has been said concerning the preventive and curative aspects of the intra-oral types of cancer. To a great extent, the problem of prevention is dependent upon (1) the will and ability of the individual to seek adequate dental care, and (2) on the dental profession to provide this care. The fact that young women are more apt to secure dental care (*5, p. 457; 6, p. 632*), presumably for the sake of appearance, may in part explain the fact that the mortality rate from cancer of the buccal cavity is more than six times higher among men. However, because of the low mortality rate from cancer of the buccal cavity among females, little significance can be attached to the differences between spinsters and other women.

CANCER OF THE STOMACH

For all ages 15 years and over, combined, the rate of mortality from cancer of the stomach for married men was 5 percent greater than for single men. A difference, possibly significant, was observed after 75 years of age, when the rate among married men continued to rise while that for single men seemed to level off (fig. 5). Married women, however, had a 20 percent higher rate than spinsters. This more favorable rate for single women was especially marked for the most important years of life, 35 to 74, when the rate for single women averaged 25 percent lower than that for married women.

CANCER OF THE PERITONEUM AND DIGESTIVE TRACT (OTHER THAN THE STOMACH)

No appreciable difference in mortality from cancer of the peritoneum and digestive tract (other than the stomach) was apparent between single and married women (fig. 6). This was unlike the relationship found for cancer of the stomach. However, for these sites also, at ages 55 to 74, the rates for

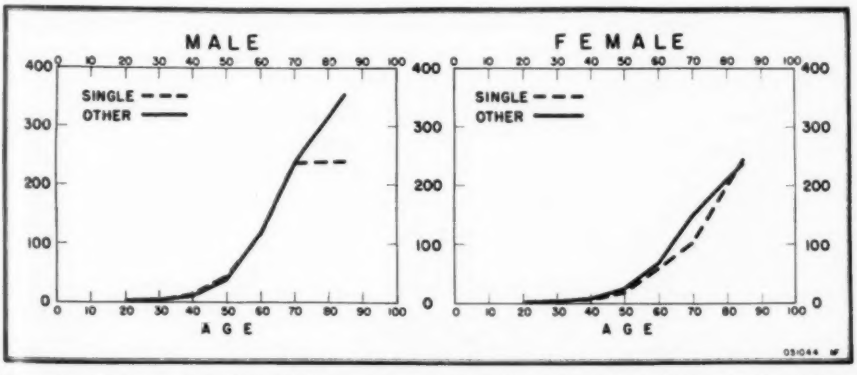

FIGURE 5.—Cancer of the stomach, by sex and age according to marital status, death rates per 100,000, white population, city of New York, 1939–41

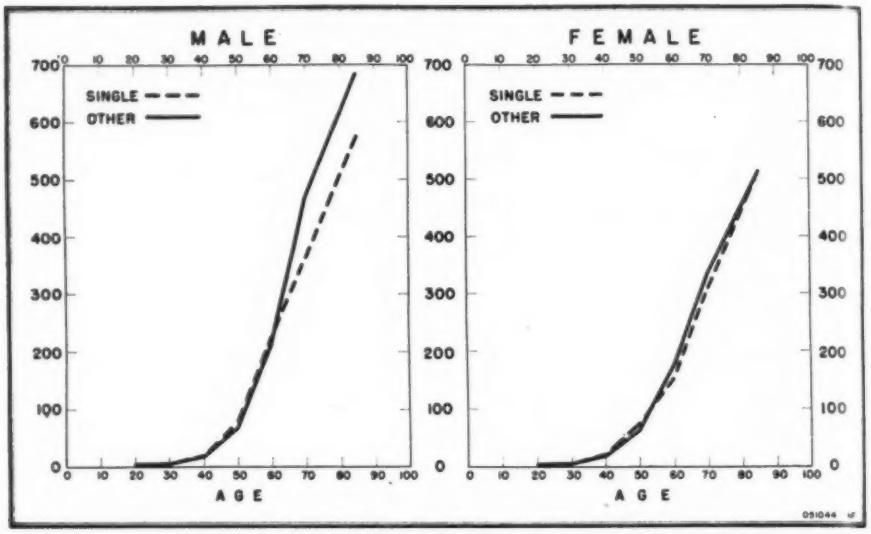

FIGURE 6.—Cancer of the peritoneum and digestive tract (other than the stomach) by sex and age according to marital status, death rates per 100,000, white population, city of New York, 1939-41.

the married were in excess of those for single women.

By contrast, the rate for males (aged 15 and over) who had married was 11 percent higher than for bachelors, the difference being as much as 24 percent after age 65. Men who had remained married as well as single men were found to have approximately similar rates at all ages. The adverse rate after 65 for those who had married resulted from the higher rate among those who had been widowed or divorced. Possibly the man who has become accustomed to a regulated way of living finds it most difficult, after the loss of his wife, to maintain a balanced routine when old than does the man who has lived alone all through life.

CANCER OF THE RESPIRATORY SYSTEM

During 1939–41 the death rate from cancer of the respiratory system among males was approximately four times as

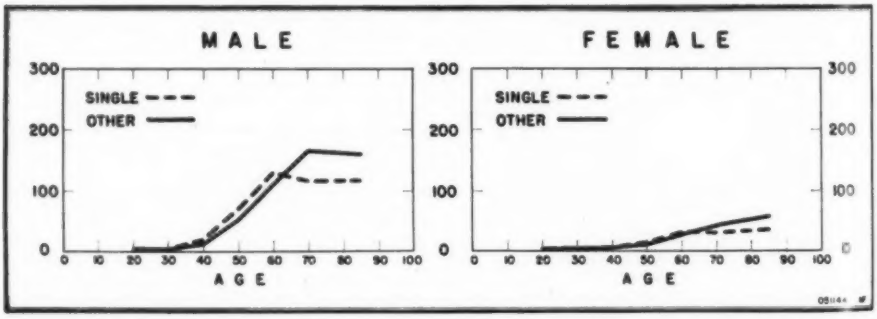

FIGURE 7.—Cancer of the respiratory system by sex and age according to marital status, death rates per 100,000, white population, city of New York, 1939–41.

high as that among females. In both sexes, the mortality rates for single and others were only slightly (and not significantly) different. It is interesting that the rate for all ages (15 years and over) was 6 percent higher for the single among males but exactly that much less among females. Of interest, too, is the fact that for both men and women the rate was higher for single persons at ages under 65 years but lower than the rate for married persons after 65 (fig. 7).

ALL OTHER CANCERS

Because of the small numbers involved, we have grouped the mortality reported for cancers of all specified organs not already discussed, together with that for cancer of the unspecified organs. Deaths from cancer of the skin, the brain and other parts of the nervous system, those grouped in the International Classification as of "other and unspecified organs," as well as cancer of the breast among males (of which only 29 deaths were reported for the 3 years), and of the urinary organs among females total 1,536 for males and 1,760 for females among persons 15 or more years of age.

Figure 8 shows that for both males and females the rates were higher for the single, and the greatest differences between the rates for the single and those for others occurred after 65 years of age. Because of the miscellaneous nature of this grouping, little significance can be attached to the fact that for all ages over 15 bachelors had a mortality rate 29 percent higher than men who had married, while spinsters showed an excess of 7 percent over other women.

Inasmuch as the data for this miscellaneous group do not include comparable sites for the two sexes, comparison of the rates for men and women has no meaning.

SUMMARY AND CONCLUSIONS

There is evidence from the data analyzed that cancer mortality bears some relation, direct or indirect, to marital status. For some of the site groups, the lower death rates for those who had married appear to be statistically significant; for example, the breast and genital organs (other than the uterus) among females, and the buccal cavity among males. On the other hand, spinsters appear to have lower mortality from cancer of the uterus and of the stomach, and bachelors from cancer of the peritoneum and digestive tract (other than the stomach).

For other site groups, the evidence is not conclusive. This may be due to the fact that marital status has no bearing on cancer in these sites. Then, too, our findings may be affected by the fact that the rates for the single at the older ages are based upon relatively small

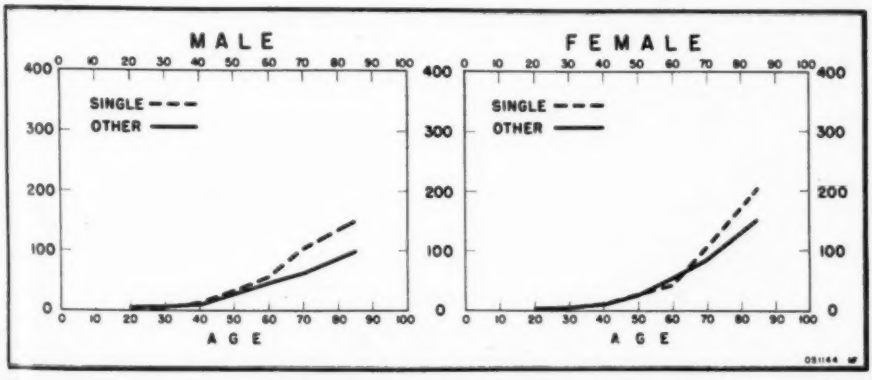

FIGURE 8.—All other cancer, by sex and age according to marital status, death rates per 100,000 white population, city of New York, 1939–41.

populations and that marital status for older persons may not be accurately reported on death certificates and census schedules.

While our findings are in general agreement with similar data for England and Wales and for Australia, it would nevertheless appear desirable that the analysis of the relationship of cancer mortality and marital status be studied further; although it is hoped that public-health and research workers may gain some assistance from the data which we have presented.

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PANETH CELLS IN CARCINOMAS OF THE SMALL INTESTINE IN A MOUSE AND IN A RAT

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INTRODUCTION

Carcinomas of the small intestine are rare tumors in man, and spontaneous tumors of this type are also very rare in laboratory animals. We have had an opportunity, however, to study two induced tumors at this site, one in a mouse and one in a rat, which arose following treatment with a carcinogen. The carcinoma in the mouse contained a strikingly large number of Paneth cells, and while only a few such cells were found in the tumor of the rat, they were unmistakably a part of the new growth. Since the origin and function of these cells are still uncertain, a description of their appearance in neoplasms may throw some light on these questions. No report of the occurrence of such cells in tumors of man could be found in the literature.

Adenocarcinomas of the small intestine in the mouse were briefly described by Bashford (*1*, *2*) He did not refer to Paneth cells. More recently Lorenz and Stewart (*3*) described the development and morphology of carcinomas of the small intestine which occurred in mice fed dibenzanthracene or methylcholanthrene. They wrote that "many of the cells were filled with highly refractive acidophilic granules resembling those normally found in the cells lining the crypts of Lieberkühn in the intestinal mucous membrane."

No previous report on carcinomas of the small intestine of the rat was found.

MATERIALS

The carcinoma in the mouse developed in a strain A backcross aged 18½ months that had received a methylcholanthrene-olive oil emulsion with acid pepsin in the drinking water for 13 months. Twelve lung tumors, multiple papillomas of the forestomach, and atrophy of the testicle were also found; these lesions occur characteristically in mice receiving methylcholanthrene. The carcinoma of the small intestine was found 17 cm. from the pylorus and appeared as a mass about 1 cm. in diameter arising from one side of the digestive tube. It was attached by adhesions to the anterior abdominal wall. An abscess cavity about one-third centimeter in diameter was included in the tumor. The whole mass of tissue was taken for section.

The general architecture of the tumor

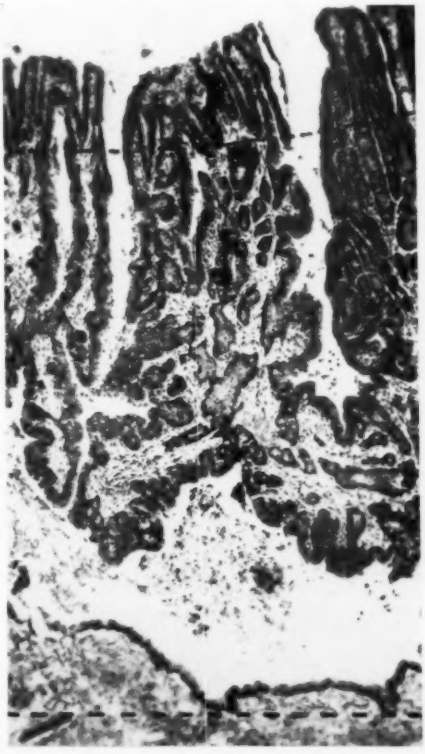

FIGURE 1.—General architecture of the tumor of the small intestine. Portion where Paneth cells are most frequent lies between dotted lines. Hematoxylin and eosin stain. ×75.

is shown in figure 1. A distorted reproduction of the intestinal glands and villi was observed with mucosal projections covered by common epithelial cells adjacent to the intestinal lumen, and extensions of the neoplastic tissue into the musculature. No metastases were found, and the general morphology of this carcinoma corresponded to descriptions and photographs given by Lorenz and Stewart (*3*) for a large group of these tumors.

The Paneth cells were most abundant in the basal portions of the neoplastic glands, as indicated in figure 1. By maintaining this location, similar to that of the normal intestinal gland, the tumor suggests an organ reproduction rather than a single-cell reproduction, a condition sometimes observed in other tumors of the mouse (*4*). The Paneth cells in the neoplastic mucosa were interspersed with cells resembling the common epithelial cells and occasional goblet cells.

All the tumor material had been fixed in Zenker-formol, and consequently the number of special stains which could be applied was limited. The De Galantha stains (*5*) especially recommended by Hertzog (*6*) were used, and also the three silver impregnation and counterstaining methods described by Popoff (*7*). When Popoff's methods I and II

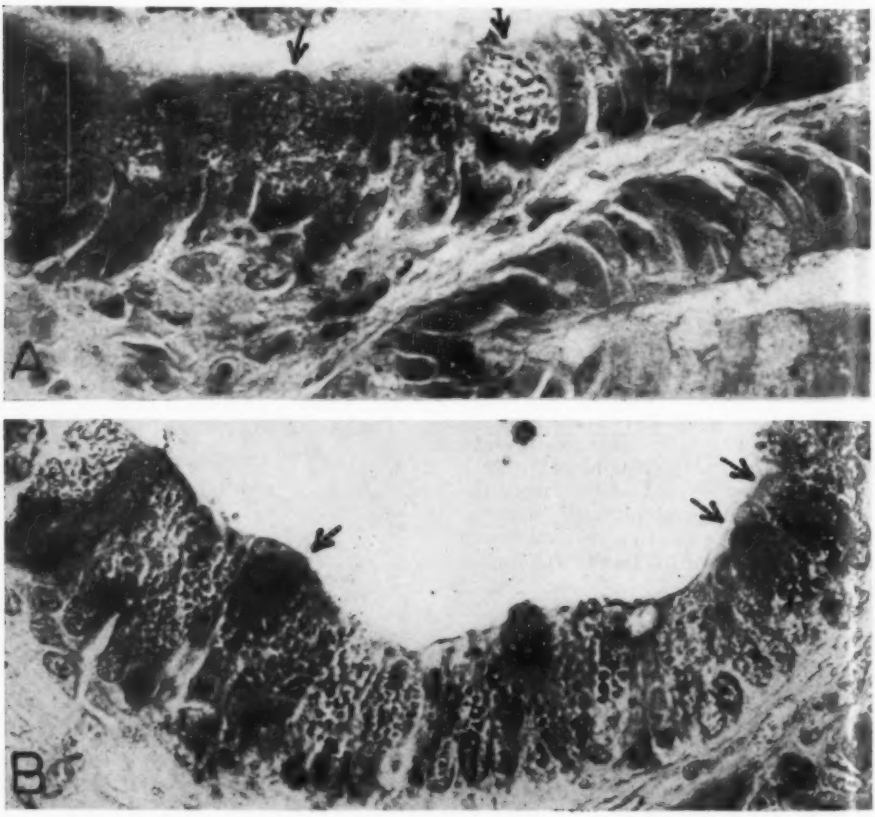

FIGURE 2.—Paneth cells stained by method III of Popoff. *A*, Cells (marked by arrows) containing crescentic or filamentous inclusions; *B*, Cells (marked by arrows) containing large rounded granules.

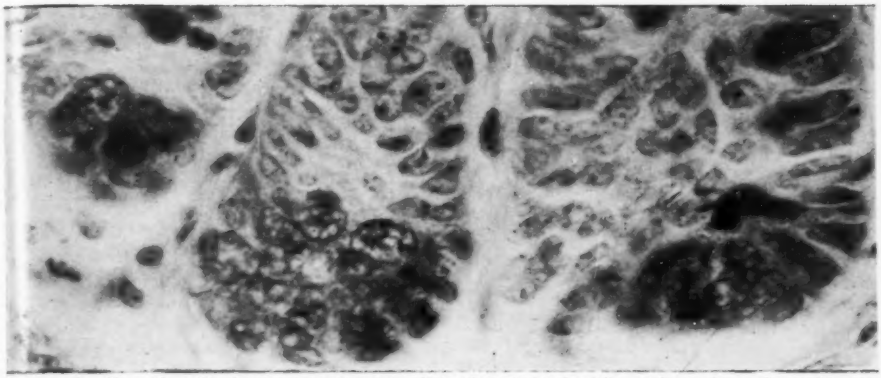

FIGURE 3.—Paneth cells from normal mouse intestine stained with mucicarmine. Granules are unstained, while surrounding cytoplasm is faintly pink. Some heavily stained cells in adjacent portions of pland are also seen.

were used, the granules showed an indistinct blue to dark blue-green color. When method III was employed, the granules showed a variety of brilliant and distinctive colors. Popoff calls this last method the potassium bichromate-eosin-silver-water blue method, and when used by him on the small intestine of the rabbit, the Paneth granules stained either blue, red, or black. When our tumor sections were treated by this method, large circular granules, which often filled the cells, took a purple or mulberry-colored stain. Other cells showed filamentous or crescent-shaped inclusions, which characteristically took an orange-red stain. Besides these, there were clear cells in which very fine granules without distinctive coloring could be seen. These finely granular cells were difficult to distinguish from goblet cells (fig. 2, *A*, *B*.) Stages which may be interpreted as different periods in the development of the Paneth cell were thus readily made out in this neoplasm and are seen more clearly here than in normal tissue.

Sections of 10 tumors from the small intestine, which are on file at the National Cancer Institute, were examined, and in 7 Paneth cells were found. These often occurred in epithelial-lined cysts which were clearly tumor extensions into the musculature or mesentery, and these cells could not be mistaken for preexisting Paneth cells included in the tumor growth. One of these tumors had been successfully transplanted and has been carried for 5 years; no Paneth cells are present in the transplant. Another group of 6 tumors derived from the cecum and having histologic features similar to the small intestine tumors were examined, and no Paneth cells found.

Special stains were used to demonstrate Paneth-cell granules in the intestine of the normal mouse. Popoff's method III showed them as orange-red granules and De Galantha's mucin stain as brilliant green refractile bodies. With Mayer's mucicarmine stain, the Paneth cells had a foamy appearance (fig. 3) since the granules proper appeared unstained, while the surrounding cytoplasm was faintly pink. This peculiar staining reaction is paralleled by Hertzog's observation that when Paneth cells were treated with a combination of De Galantha's amyloid stain and mucicarmine, the granules took a specific differential green color, and in addition red-staining droplets of mucus were present near the distal end of the cell. Bowie's pepsinogen stain (*8*), which we have modified slightly for use in mouse tissues,[1] proved to be a very

[1] Tissues to be stained by the Bowie method were kept in a dark cold room. The Regaud fixative was changed every day for 4 days. The tissue was then put in 5-percent aqueous potassium bichromate for 4 days longer without changing the fluid and was then washed 24 hours in running water.

FIGURE 4.—Paneth cells stained by Bowie's pepsinogen method. Granules are selectively stained a dark purple color.

satisfactory stain for demonstrating pepsinogen granules in the stomach; and it was discovered that Paneth cells in the duodenum and ileum were also selectively stained (fig. 4), although the color was darker and less brilliant. In taking sections of the ileum of the normal mouse for these special staining procedures, a small piece of pancreas was always included. It was noted that the pepsinogen stain was the only one which also selectively stained elements of the pancreatic acinar cells, namely, the zymogen granules. This suggests the probability that the Paneth cell granule contains a zymogen substance. In addition, there are other substances in the Paneth cell granule which give a positive staining reaction with the De Galantha stains and Popoff method III, and some substance in the cytoplasm of the Paneth cell which gives a positive stain with Mayer's mucicarmine, but these last reactions do not occur with pancreatic acinar cells.

The carcinoma of the rat occurred in a 14-month-old male of the Buffalo strain which received N-acetyl-2-aminofluorene in the diet. A complete account of this experiment is to be published later. The tumor had extended into the mesentery and pancreatic tissue. It was in the infiltration into the pancreas that a few Paneth cells were observed with the hematoxylin-eosin stain; they were interspersed with the usual epithelial-cell types lining the cystlike glandular structures of the tumor (fig. 5).

DISCUSSION

Two recent authors, Hertzog (*6*) and Popoff (*7*), have discussed the nature of Paneth cells and have arrived at different conclusions. Hertzog's opinion is that the Paneth cells give evidence of being mucoid in character rather than independent zymogenic cells, while Popoff believes that they are secretory zymogenic cells, entirely different from the argentaffin cells which he considers as representing a process of rejuvenation of functionally exhausted mucous cells. Mols (*9*), who particularly studied Paneth cells in the mouse, considered them to be typical serous cells; and he believed that they went through definite stages from one where they appeared as ordinary epithelial cells to the condition in which they were filled with granules. He observed that Paneth cells were most frequent at 18 cm. from the pylorus,

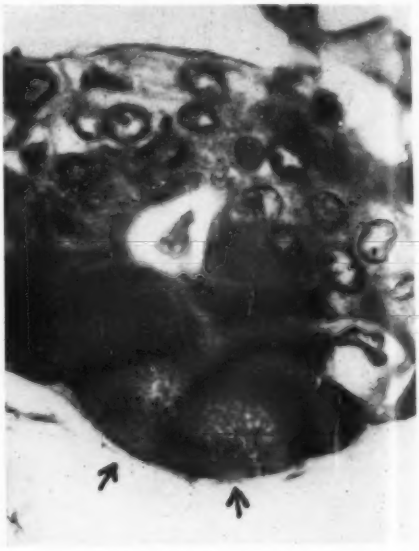

FIGURE 5.—Paneth cells in tumor of small intestine in the rat. Hematoxylin and eosin stain. × 1260.

and he never observed these cells in mitosis.

Nicolas (*10*) studied the epithelium of the small intestine in a number of mammals and gave detailed descriptions of the appearance of Paneth cells in the mouse. He described a type of granule taking a safranine stain which he considered as peculiar to certain stages of Paneth-cell development in the mouse, since he found it in no other animal. The stages which he describes, from an early indifferent cell with clear cytoplasm to one with crescentic bodies, and finally to one completely filled with large granules, appear to be reproduced in the carcinomatous cells (fig. 2, *A*, *B*). Nicolas believed that Paneth cells were not destroyed, but having arrived at the end of their secretory cycle, they returned to their original state. Klein (*11*), who later studied them, concluded that these cells corresponded in structure and microchemical reactions to the enzyme-producing cells of other glandular organs. He noted also that granules were commonly found which were composed of a faintly stained central mass with a deeply staining crescent at one border, but he considered these forms as due to imperfect fixation and thought that they had no previous existence in the living cell. Bensley (*12*), who also was concerned with these crescent-shaped masses, thought they were probably due to imperfect fixation. He considered them an indication either of a twofold chemical nature of the granules or of a twofold secretory activity of the cell and stated that these appearances were to be given a chemical interpretation rather than an architectural one. In our tumor sections stained by method III of Popoff, this appearance of crescent or rod-shaped bodies is obvious and occurs most often in cells taking the orange-red stain, so they may represent a definite chemical and morphologic development of the cell.

Our observations on the normal mouse intestine and on tumors derived from the small intestine lend support to the opinion that Paneth cells do not represent a unique cell type, independent of other epithelial cell types in the mucosa of the small intestine. They can be found along with epithelial and goblet cell forms in early neoplastic processes. Perhaps their relatively high degree of differentiation precludes their continued participation; and therefore they have not been observed in tumors of man, where the earliest neoplastic change is seldom seen.

SUMMARY

The morphology and staining reactions of Paneth cells in two carcinomas of the small intestine, one in a mouse and one in a rat, are described. The cells constituted an actual part of the neoplasms and showed developmental stages not often identified in normal tissue.

A number of staining reactions of Paneth cell granules in the normal mouse intestine were investigated. The granules were selectively stained by Popoff's method III, by De Galantha stains, and by the Bowie pepsinogen stain. Mayer's mucicarmine stain left the granules uncolored while the surrounding cytoplasm was faintly pink. The pepsinogen stain was the only one which also gave a selective staining to granules in the acinar cells of the pancreas.

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EFFECT OF NUCLEATES ON DEHYDROGENASE SYSTEMS[1]

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INTRODUCTION

The metabolism of each living cell is an inherent property of the cell and is controlled by both genetic and environmental factors. The influence of environmental factors, i. e., nutrition, toxins, accelerators, inhibitors, etc., has long been known. In contrast, studies of the genetic influence on cell metabolism have been relatively sparse and until recently have not been decisive. (See (*3*) for earlier literature on adaptive enzymes.)

Within the past 4 years, Lindegren (*4*) and Spiegelmann (*5*) in their studies on yeast, and Tatum, Beadle, and their collaborators (*6*) in their studies on the mold *Neurospora* have adduced clear-cut evidence for the control of specific enzymatic functions by the genetic constitution of the cell. Mutations were induced in these organisms by various techniques. These mutations led to altered enzymatic functions which were inheritable in successive generations, and these mutations followed in general a Mendelian distribution. The work of Avery, MacLeod, and McMarty (*7*) on the transformation of the pneumococcal types by means of specific desoxypentosenucleic acid has focused attention on the importance of nucleic acids in cell mutations.

It seems probable, therefore, that the control of enzymatic activity may be in part a property of the gene. It may be further assumed that the genic material exerts this control not remotely but by specific kinds of chemical interaction with the enzymes directly concerned. A considerable amount of evidence exists which links the nucleic acids with gene activity (*8*, *9*); indeed, the gene may be nucleoprotein in nature (*10*). The type of nucleic acid associated with the gene is desoxyribosenucleic acid (thymus type). More closely associated with the cytoplasm is ribosenucleic acid (yeast type). Because of the possibility that cytoplasmic factors may be concerned with heredity (*11–13*), the influence of the latter type of nucleic acid cannot be omitted from consideration.

As a direct chemical approach to the study of the influence which genic material may have on the metabolism of the cell, investigations have been made of the effect of the addition of ribosenucleate and of desoxyribosenucleate on the activity of enzymes in vitro (*1*, *2*). Of a number of enzymatic systems studied, the dehydrogenase systems were the most strikingly affected by the nucleates. The results of this approach are described in detail in the present paper. During the course of study, it was observed that certain tissues possessed the capacity to desaminate the nucleates while the latter were still in the state of polymerized tetranucleotides; the enzymes responsible for the desamination, hitherto undescribed, were discussed in an earlier paper (*14*). The possibility that several desaminated forms of the nucleates may be involved in the phenomena described herein has been considered. Despite its length, the work is in no sense complete. There have been few if any comparable studies in the literature to inform or guide the authors.[2] Much remains to be done before the phenomena described may be entirely interpretable.

EXPERIMENTAL PROCEDURE

The dehydrogenase systems in tissues may be considered to act according to

[1] Preliminary accounts of this investigation have been published (*1*, *2*).

[2] The only study in the literature which is concerned with an extended experimental investigation of the equilibria in dehydrogenase systems is that of Dixon and Thurlow (*15*) on xanthine dehydrogenase. The use of nucleates as possible substrates in the dehydrogenase systems has been briefly considered by Von Euler and Skarzynski (*16*) and by Tsuge (*17*).

the following scheme, where SH_2 is the subtrate and D the dehydrogenase:

$$SH_2 + D = SH_2.D \qquad (1)$$

The first step is a combination of enzyme and substrate in which the available hydrogen of the substrate becomes labile and in the presence of a suitable acceptor A leaves the substrate (oxidation) and combines with the acceptor (reduction).

$$SH_2.D + A = S + D + AH_2 \qquad (2)$$

In the presence of any number of hydrogen acceptors, $A_1, A_2 \ldots . A_n$ and of dehydrogenases $D_1, D_2 \ldots . D_n$ the hydrogen originally from substrate SH_2 may be transported from one acceptor to the next until it reaches the ultimate acceptor in aerobic organisms, namely, oxygen.

The dehydrogenases are essentially protein in nature. The acceptors, as far as they are known, are not protein in nature and include such substances as thiamine phosphate, coenzymes I and II (di- and triphosphopyridine nucleotides), riboflavinphosphate, etc. For the experimental determination of specific dehydrogenase activity, systems may be set up in vitro which take any one of these factors into account. Where an over-all estimation of dehydrogenase activity is desired, it has been found that for most cases the sum function of the natural acceptors can be imitated by the dye, methylene blue. Thus dehydrogenase activity can be observed in the system substrate-dehydrogenase-methylene blue in which the dye functions as a ready acceptor of hydrogen. Under such conditions, the dye is reduced to the colorless leuco form. Inasmuch as this leuco form is readily autoxidizable, the system must be investigated under strictly anaerobic conditions. The rate at which the system approaches the leuco form of the dye is therefore a measure of the dehydrogenase activity of the system.

Several experimental procedures have been devised in the past to measure dehydrogenase activity (*18*). Most of these have been based upon the work of Thunberg, who pioneered in this field. The essential point in the technique involves the removal of oxygen from the apparatus, and this was in general accomplished either by evacuation through the vacuum pump or by filling the evacuated apparatus with oxygen-free nitrogen.

The authors found after many trials that the available apparatus on the market was inadequate for their needs and that most of the techniques used hitherto were not satisfactory for their purposes. Measurements of relatively high precision and of reproducibility among replicate determinations of only a few percent were desired. At the same time, it was desired to set up daily a great many experimental determinations some of which might be of long duration. Leaks in the apparatus were therefore to be avoided, and after many attempts the apparatus described in figure 1 was designed and gave highly satisfactory use.

Figure 1 shows that solutions of the reaction components must be introduced through the bore of the stopcock. For

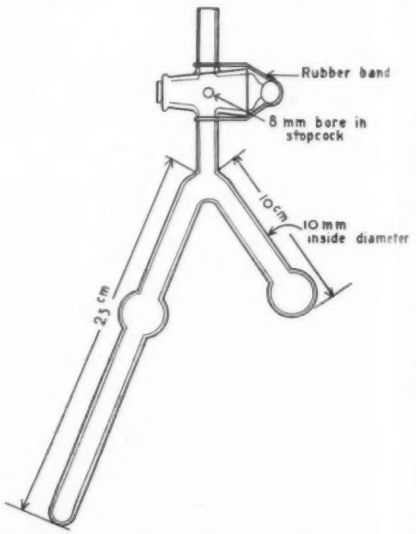

FIGURE 1.—Tube used for dehydrogenase experiments.

this purpose, pipettes delivering a volume of 1 cc. were made from pyrex tubing of appropriate width and were calibrated before use. The technique is as follows: A designated volume of chilled tissue extract is introduced into the long arm of the tube after tilting the latter at the appropriate angle. Designated volumes of other reactants are then added. The entire tube is then turned through 180° and the dye solution introduced into the short arm of the tube. The outside of each pipette prior to introduction into the tube is carefully wiped with cloth to remove adhering solution. It has been found advantageous in pipetting to place the tip of the pipette against the crotch of the two arms of the tube. When all the reactants are added to the tube, the latter is connected with a high vacuum pump and the residual air evacuated with gentle agitation at 12–18 mm. mercury for 2 minutes. At the end of this period, the stopcock is closed and tightened against the barrel with strong rubber bands. The use of these bands is essential. Each tube in a series of determinations is treated in this fashion. When the last tube is closed, the entire lot of tubes is simultaneously tipped so that the dye solution mixes with the solutions of substrates, extract, etc. The tubes, after thorough mixing of the contents, are set in a water bath, and the time of decrease in the color is observed.

The end point of the reaction was chosen as follows: The decolorization of methylene blue falls off asymptotically (*1*); for accurate estimation, some time short of complete decolorization must be adopted as an end point. Standards of the tissue extracts diluted with water to the designated volumes were therefore prepared and treated with sufficient methylene blue to correspond to a decolorization of the dye amounting to 98 percent. The color of this small amount of dye naturally fades rapidly even in the presence of air. The shaking of this standard vigorously in the presence of air causes a return in the color, which may be used as a measure of the end point of the dehydrogenation in the test apparatus. As an alternative method, solutions of the diluted extract were treated with a solution of a permanent blue water color, which was added in just the quantity necessary to match the color of the methylene blue standard. Either procedure was found to be satisfactory.[3]

The tissues employed in these studies were excised fresh from animals killed by decapitation or cervical dislocation and were ground with sand to a fine paste in a glass mortar and then taken up in a designated volume of water. After 10 minutes' standing, with intermittent stirring, the mixture was centrifuged at 1,500 r. p. m. and the supernatant used for the experiments. It was found advisable to keep the extract chilled during the setting up of the experiments.

The nucleate preparations were purified products earlier used for physicochemical studies (*19*). The sodium yeast nucleate was prepared from Eastman yeast nucleic acid and the sodium thymus nucleate preparations were made from calf thymus according to the method of Hammarsten. Several preparations were used with entirely concordant results. In previous studies, it was shown that these preparations were nearly entirely nondiffusible through cellophane (*14*). The xanthine and other purines studied were Hoffmann-LaRoche products. The solutions of the purines were prepared as described previously (*14*). The methylene blue used was an Eastman product.

RESULTS

REPRODUCIBILITY OF DATA

A number of experiments were set up in order to determine the reproducibility of replicate determinations. A

[3] Many workers have used an end point corresponding to 50 to 20 percent decolorization of the dye. This procedure is satisfactory for very dilute and nearly transparent mixtures. It was not used in the present investigation for two reasons: (1) The mixtures used were too opaque to determine the end point with sufficient sharpness; and (2) the use of many of the reactants, such as the nucleates, causes a change in the color of the dye, hence for each reacting mixture a separate end-point standard must be set up. At the 98-percent decolorization point, the different reacting solutions have the same color, and a single end-point standard is sufficient.

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sample is given in the following tabulation. In all cases the variation of the end-point determination from one tube to another was never more than ±5 percent from the mean and was usually much less. We attribute this reproducibility in part to the lack of air leaks in the tubes employed and to the use of accurately calibrated pipettes.

Replicate determinations of reduction capacity for methylene blue of a tissue extract [1]

Tube No.	*Decolorization rate* (*Minutes*$^{-1}$ × 10^4)
1	227
2	243
3	227
4	233
5	227
6	222

[1] Mixtures composed of 1 cc. rat-liver aqueous extract (160 mg. tissue), 1 cc. methylene blue (1.24×10^{-5}M), and 1 cc. distilled water. Temperature, 22° C.

NUCLEATES IN MOUSE-TISSUE EXTRACTS

The effect of yeast nucleate and of thymus nucleate on the decolorization rate of methylene blue is shown in table 1. [4] Under the experimental conditions employed, the rate of decolorization was reduced in the presence of the nucleates, the reduction in rate being greater in the case of thymus than of yeast nucleate. [5] Data, at present incomplete, indicate that the relative activity for the tissues in descending order is liver, kidney, brain, spleen, and muscle.

EFFECT OF ADDED XANTHINE AND OF YEAST NUCLEATE WITH VARYING AMOUNTS OF DYE

Table 2 shows the effect of added xanthine and yeast nucleate with varying amounts of dye. At very low concentrations of the dye the added xanthine or nucleate retarded the decolorization rate, but at higher concentrations of dye the added xanthine or nucleate accelerated the decolorization rate. At lower dye concentrations, the effect of adding nucleate together with xanthine was a depressing of the decolorization rate below that characteristic of that of xanthine alone. Only at the highest dye concentration used did the mixture of xanthine and nucleate cause an accelerating rate of decolorization over that of the xanthine

[4] For brevity, in the tables yeast nucleate and thymus nucleate are referred to as YNA and TNA, respectively.

[5] Mixtures of methylene blue with thymus nucleate are not blue but green. Spectrophotometric studies of mixtures in the proportions given in table 1 revealed that there was a shift of about 50A in the peak of maximum absorption of the dye toward the red. In the presence of yeast nucleate there was a similar shift of only about 5A. Absorption studies in the ultraviolet revealed that the maximum at 2,600 A of the nucleate was not affected by the presence of the dye. The reason for the red shift of the dye in the presence of the nucleates is not known at present. This shift did not affect the reducing capacity of the dye.

TABLE 1.—*Effect of YNA and of TNA on decolorization rate of methylene blue in aqueous extracts* [1] *of tissues of strain A male mice*

Mixture	Amount	Decolorization rate in—				
		Liver	Kidney	Muscle	Brain	Spleen
	Cubic centimeters	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4
Extract	2					
Water	2	135	500	21	147	37
Methylene blue	1					
Extract	2					
Water	1.5					
YNA	.5	125	416	15	81	35
Methylene blue	1					
Extract	2					
Water	1.5					
TNA	.5	49	200	12	47	32
Methylene blue	1					

[1] Stock solutions were equivalent per cubic centimeter, respectively, to 83 mg. liver, 250 mg. kidney, 333 mg. muscle, 250 mg brain, and 333 mg. spleen; methylene blue concentration, 1.24×10^{-5}M; YNA, 1 percent; TNA 1 percent.

TABLE 2.—*Effect of xanthine and of YNA on methylene blue decolorization by aqueous extract[1] of the livers of male rats[2]*

Methylene blue (millimols)	Xanthine	YNA	Decolorization rate
	Millimols	*Mg.*	*Minutes*$^{-1}$ × 10^4
0.12	0	0	6,667
	1.6	0	5,000
0.31	0	0	2,000
	1.6	0	1,818
	0	5	1,250
	1.6	5	1,429
	0	10	769
	1.6	10	952
0.93	0	0	476
	1.6	0	588
	3.2	0	500
	4.8	0	455
1.24	0	0	123
	1.6	0	400
	0	5	200
	1.6	5	303
	0	10	167
	1.6	10	263
	0	30	101
2.48	0	0	29
	1.6	0	141
	0	5	89
	1.6	5	117
	0	10	85
	1.6	10	105
	0	20	81
4.96	1.6	0	43
	1.6	5	38
9.92	1.6	0	1
	1.6	5	5

[1] Extract equivalent to 82 mg. tissue per cubic centimeter. Mixtures composed of 2 cc. extract and sufficient water added in addition to 1 cc. dye and 1 cc. substrate to make total volume 6 cc. Temperature, 24° C.; pH, 7.1. Anaerobic conditions maintained throughout by employment of tubes evacuated and sealed at 18 mm. mercury. End point at 96-percent decolorization.

[2] Concentrations given are of stock solutions.

alone. It is clear that in the presence of xanthine, if nucleate is to have an accelerating effect on the decolorization rate, it is necessary to use relatively high dye concentrations. With increasing nucleate concentration, the depressing effect is increased.[6] The points mentioned are considered individually in the following sections.

APPARENT XANTHINE DEHYDROGENASE ACTIVITY

Xanthine dehydrogenase activity is formally defined as the difference between the decolorization rate in the presence of this substrate and the decolorization rate in its absence. On this basis, data derived from those in table 2 are given in table 3.

[6] Tsuge (*17*) reported that at low dye concentrations in rabbit-tissue extracts the addition of yeast nucleate accelerated the decolorization rate. We have been unable to confirm this finding after repeating Tsuge's experiments exactly.

TABLE 3.—*Apparent xanthine dehydrogenase activity in rat-liver extract*[1]

Methylene blue (millimols)	Xanthine	YNA	Activity
	Millimols	*Mg.*	*Minutes*$^{-1}$ × 10^4
0.124	1.6	0	−1,667
0.31	1.6	0	−182
	1.6	5	+179
	1.6	10	+183
0.93	1.6	0	+112
	3.2	0	+24
	4.8	0	−21
1.24	1.6	0	+277
	1.6	5	+103
	1.6	10	+96
2.48	1.6	0	+112
	1.6	5	+28
	1.6	10	+20

[1] Values for apparent activity=Velocity in water+xanthine or in YNA+xanthine−Velocity in water or in YNA, respectively.

It is evident that xanthine dehydrogenase activity as defined is a function of the dye concentration as well as that of added yeast nucleate, and for that matter of the concentration of xanthine (*15*) (table 3). At low concentrations of the dye the activity was negative (*1*); but when yeast nucleate was present at these concentrations, the value for the activity became positive in sign, i. e., there was an accelerating effect. Thus although either xanthine or yeast nucleate alone produced a depressant effect on the decolorization rate of the dye (at lower concentrations of the dye), the two substances together produced an accelerating effect on the rate. At higher concentrations of the dye, the xanthine dehydrogenase activity was increasingly positive, appeared to reach a peak of activity, and then fell off again. At such higher dye concentrations the effect of added nucleate was to depress the decolorization rate. Finally at a single concentration of dye the effect of increasing concentration of xanthine was to depress the dehydrogenase activity. It is clear now why the adjective "apparent" was used to describe the xanthine dehydrogenase observed herein.

EFFECT OF ADDED YEAST NUCLEATE

Further data in table 2 were extracted and recalculated and are given in table 4. These data indicate the

TABLE 4.—*Effect of YNA on decolorization velocity in rat-liver extract in the presence and the absence of added xanthine*

Methylene blue (millimols)	YNA	Change in velocity of decolorization		
		No xanthine [1]	Xanthine [2]	Xanthine +YNA [3]
	Mg.	*Minutes*$^{-1}$ ×10^4	*Minutes*$^{-1}$ ×10^4	*Minutes*$^{-1}$ ×10^4
0.31	5	−750	−389	−571
	10	−1,231	−866	−1,048
1.24	5	+77	−97	+180
	10	+44	−137	+140
	30	−22		
2.48	5	+60	−24	+88
	10	+56	−36	+76
	20	+52		
4.96	5		−5	
9.92	5		+4	

[1] Values=Velocity in presence of YNA−Velocity in water alone.
[2] Values=Velocity in presence of xanthine+YNA− Velocity in xanthine alone.
[3] Values=Velocity in presence of xanthine+YNA−Velocity in water alone.

effect on the velocity of decolorization of the dye in the presence of added xanthine and of added nucleate. They differ from those in table 3 in that differences were calculated between other quantities. These reveal that in the presence of nucleate the effect of increasing nucleate concentration at each level of dye concentration resulted in a decrease in the decolorization rate of the dye. With nucleate alone, the effect at low dye was depressant on the rate, accelerant at higher dye, and depressant again at still higher dye concentrations, i. e., there seemed to be a maximum in the effect of the nucleate on the rate. At higher nucleate concentrations, this maximum was shifted in the direction of higher dye concentrations. When xanthine was present, this maximum was shifted to still higher dye concentrations.

XANTHINE AND NUCLEATES IN EXTRACTS OF RAT LIVER AND OF HEPATOMA

Studies such as those described in table 2 were extended to comparisons of normal liver and the transplanted rat hepatoma and included a similar comparison of yeast and thymus nucleates in extracts of these tissues from male Osborne-Mendel rats. The data (table 5) are graphically presented in figure 2. These data are representative of a large number of experiments involving many samples of the tissues concerned. The results in all cases were completely concordant.

At extremely low dye concentrations, it was impossible to measure the decolorization rates in the liver extracts because of their magnitude, and it was impracticable to measure the decolori-

TABLE 5.—*Decolorization velocity of methylene blue in aqueous extracts of normal liver and of transplanted hepatoma 31 in male Osborne-Mendel rats* [1]

Methylene blue (millimols)	Xanthine	YNA	TNA	Decolorization rate	
				Liver	Hepatoma
	Millimols	*Mg.*	*Mg.*	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4
0.016	0	0	0		450
	0	2.5	0		10,000
	0	0	2.5		400
	0	0	0		178
0.031	0	2.5	0		5,000
	0	0	2.5		222
	1.6	0	0		230
	1.6	2.5	0		5,000
	1.6	0	2.5		2,500
0.062	0	0	0		56
	0	2.5	0		3,333
	0	0	2.5		164
	1.6	0	0		55
	1.6	2.5	0		3,333
	1.6	0	2.5		1,428
0.155	0	0	0	5,000	24
	0	2.5	0	2,500	1,666
	0	0	2.5	556	62
	1.6	0	0	3,333	33
	1.6	2.5	0	2,500	2,000
	1.6	0	2.5	1,250	909
0.31	0	0	0	2,000	10
	0	2.5	0	1,429	833
	0	0	2.5	333	36
	1.6	0	0	1,667	20
	1.6	2.5	0	1,429	769
	1.6	0	2.5	667	58
0.62	0	0	0	714	5
	0	2.5	0	667	370
	0	0	2.5	294	23
	1.6	0	0	769	11
	1.6	2.5	0	667	222
	1.6	0	2.5	476	24
1.24	0	0	0	218	
	0	2.5	0	218	
	0	0	2.5	111	
	1.6	0	0	250	
	1.6	2.5	0	227	
	1.6	0	2.5	178	
2.48	0	0	0	39	
	0	2.5	0	91	
	0	0	2.5	48	
	1.6	0	0	121	
	1.6	2.5	0	111	
	1.6	0	2.5	105	
4.96	0	0	0	4	
	0	2.5	0	66	
	0	0	2.5	18	
	1.6	0	0	14	
	1.6	2.5	0	18	
	1.6	0	2.5	10	

[1] Aqueous extract of tissues equivalent to 166 mg. per cubic centimeter. Mixtures consisted of 1 cc. extract, 1 cc. dye, 1 cc. water or substrates so that total volume was 3 cc. Temperature, 25° C. Anaerobic conditions maintained throughout by employment of tubes evacuated and sealed at 18 mm. mercury. End point at 98-percent decolorization; pH 7.1. Concentrations given are of stock solutions.

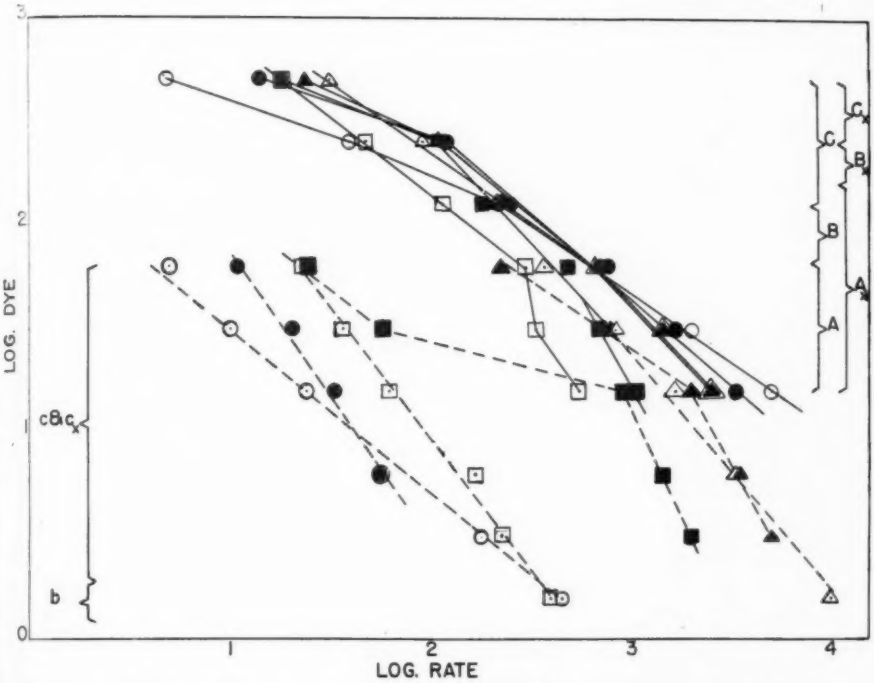

FIGURE 2.—Effect of nucleates, of xanthine, and of methylene blue on the decolorization rate of the dye in extracts of rat liver and of the transplanted rat hepatoma. Continuous lines refer to the liver, broken lines refer to the hepatoma. Open circles refer to extracts in water alone, solid circles refer to extracts plus xanthine. Open triangles refer to extracts plus yeast nucleate, solid triangles refer to extracts plus yeast nucleate plus xanthine. Open squares refer to extracts plus thymus nucleate, solid squares refer to extracts plus thymus nucleate plus xanthine. Symbols in brackets in upper right of chart refer to patterns in liver. Symbols in brackets in lower left of chart refer to patterns in hepatoma. Symbols without subscript refer to extracts without xanthine, symbols with subscript x refer to extracts with xanthine. Abscissa based on logarithms of decolorization time in minutes $\times 10^4$. Ordinate refers to logarithms of dye in millimols $\times 10^7$.

zation rates at very high concentration of the dye in hepatoma extracts in the absence of nucleates because of the length of time involved. Under similar conditions, the rate of decolorization in liver extracts was greater than in hepatoma extracts. At the lower dye concentrations in liver extracts, added nucleates retarded the rate of decolorization, thymus nucleate more than yeast nucleate, whereas at the higher dye concentrations the addition of nucleates caused an acceleration of the decolorization rate over that in the absence of the nucleates. The same comments may be made for the hepatoma extracts, except that the accelerating effect of the nucleates on the decolorization velocity was evident over a very much greater range of dye concentrations than was the case of the liver extracts, and it was necessary to go to a very low dye concentration in order to observe even a very slight retarding effect by thymus nucleate in hepatoma extracts. In no case, was there any evidence of a retarding effect by yeast nucleate in extracts of the tumor. The effect of addition of xanthine in liver extracts containing the nucleate was to shift toward higher dye concentrations that concentration of dye at which the effect of the added nucleates changed from retarding to accelerating the decolorization rate of the dye. The transition from deceleration to acceleration of decolorization required a concentration of dye low in the case of xanthine, higher for yeast nucleate, and still higher for thymus nucleate.

On inspection of the data in table 5, a certain pattern of events may be discerned. It is possible to set up three distinct patterns based upon the relative rate of decolorization of the dye in the aqueous tissue extract alone, in the extract to which yeast nucleate was added, and in the extract to which thymus nucleate was added. Pattern A may be defined as that in which the rate in the first mentioned exceeded that in the second and third; pattern B as that in which the rate in the second mentioned exceeded that of the first and third, and pattern C as that in which the second and third mentioned exceeded in rate that of the first. In summation, the patterns are expressed as follows, where "water" refers to the extract in the absence of added nucleates:

Pattern A, Water>Yeast nucleate>Thymus nucleate

Pattern B, Yeast nucleate>Water>Thymus nucleate

Pattern C, Yeast nucleate>Thymus nucleate>Water

Table 5 shows that with the exception at 0.016 mM dye which yielded a B pattern, the hepatoma extracts yielded, at all dye concentrations studied, an invariable C pattern. At dye concentrations from 0.155 to 1.24 mM, liver extracts demonstrated an A pattern, at 1.24 mM the pattern was B, while at still higher concentrations of dye the pattern was C.

There are therefore two essential differences between normal liver and the hepatoma, namely, (1) the generally higher reduction rates under identical conditions of the liver as compared with the hepatoma, and (2) the generally accelerating effect of both nucleates in the hepatoma extracts over a wide range of dye concentrations (C pattern) compared with the depressant effect of the nucleates at low dye and the accelerant effect only at high dye concentrations in the liver extracts. Perhaps a third difference between the two kinds of tissues may be added, and that is the extraordinary capacity of yeast nucleate to accelerate the decolorization rate of the dye in hepatoma extracts.

When xanthine was added to the aqueous tissue extracts and to the extracts in the presence of the nucleates, the concentrations of dye at which the patterns changed from A to B, and from B to C were shifted toward higher dye concentrations.

The phenomena described are illustrated in figure 2, and the patterns are shown by the brackets at the corners of the figure. The curves of the data given in logarithms relate only to those data at which the liver and hepatoma are comparable, i. e., between concentrations of dye at 0.155 and 0.62 mM

(table 5). Only the data for yeast nucleate in hepatoma extracts fall within the range characteristic of that of liver (dotted line with triangle points). For liver, the patterns with subscript x relating to the presence of xanthine occur at higher levels of dye concentration than the corresponding patterns in the absence of xanthine. The addition of an oxidizable substrate affects the pattern of events produced by the nucleates.

APPARENT XANTHINE DEHYDROGENASE ACTIVITY IN EXTRACTS OF RAT LIVER AND OF HEPATOMA

When xanthine dehydrogenase activity is defined as previously, it may be noted from the calculated data in table 6 that this activity is usually a function of the dye concentration and varies with the kind of nucleate present. In the absence of nucleates, the activity in liver extracts was negative at low dye and became increasingly positive at higher dye concentrations, whereas the activity in the hepatoma extracts was positive and seemingly independent of the dye concentration. In the presence of yeast nucleate, the activity in the liver extracts was null at all dye concentrations studied, whereas the activity in the hepatoma extracts diminished with increasing dye. Finally, in the presence of thymus nucleate, the activity in both kinds of extract, which activity was very high at low dye, became smaller with increasing dye concentration. At the two lowest dye concentrations, the activity of xanthine dehydrogenase in liver was highest in the presence of thymus nucleate, next highest in the presence of yeast nucleate, and lowest in the absence of either nucleate. This augmenting effect of the nucleates on the apparent xanthine dehydrogenase activity on liver was pointed out in an earlier report (*1*) and was shown clearly in the figure published in that report. In any event, what is meant by xanthine dehydrogenase activity may be a function of several variables, and any comparison of different tissues must take these variables into account.

EFFECT OF THE NUCLEATES ON THE RATE OF DYE DECOLORIZATION IN RAT LIVER AND IN HEPATOMA EXTRACTS

The data in table 7, derived and calculated from the data in table 5, indicate the effect of the nucleates alone in the various mixtures studied. Inspection of each of the columns in table 7 reveals an interesting course of events. With increasing dye concentration, the change in decolorization velocity for liver became increasingly positive, whereas the change in decolorization velocity for hepatoma became increasingly negative. These phenomena were apparently independent of the presence of xanthine. There appeared to be a maximum at lower dye concentrations in the case of thymus nucleate in the absence of xanthine in hepatoma extracts, but this was the only phenomenon of its kind.

The data as a whole indicate a significant difference between liver and

TABLE 6.—*Apparent xanthine dehydrogenase activity in aqueous extracts of normal liver and of transplanted hepatoma 31 from male Osborne-Mendel rats* [1]

Methylene blue (millimols)	Activity in—					
	Water		YNA		TNA	
	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma
	Minutes^{-1} $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$
0.155	−1,667	+9	0	+334	+694	+847
0.31	−333	+10	0	−64	+334	+22
0.62	+55	+6	0	−148	+182	+1

[1] Values = Velocity in water and xanthine, or in YNA and xanthine, or in TNA and xanthine − Velocity in water or in YNA or TNA, respectively.

TABLE 7.—*Effect of YNA and of TNA on decolorization velocity in aqueous extracts of normal liver and of transplated hepatoma 31 from male Osborne-Mendel rats*

Methylene blue (millimols)	Change in decolorization velocity in—							
	YNA				TNA			
	No xanthine [1]		Xanthine [2]		No xanthine [3]		Xanthine [4]	
	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma
	Minutes $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$	*Minutes* $^{-1}$ $\times 10^4$
0.016		+9, 550				−50		
0.031		+4, 822		+4, 770		+44		+2, 270
0.062		+3, 277		+3, 277		+108		+1, 372
0.155	−2, 500	+1, 642	−833	+1, 967	−4, 444	+38	−2, 083	+876
0.31	−577	+823	−238	+749	−1, 667	+26	−1, 000	+98
0.62	−47	+365	−102	+211	−420	+18	−293	+13
1.24	0		−23		−107		−72	
2.48	+52		−10		+9		−16	
4.96	+62		+4		+13		−4	

[1] Values = Velocity in presence of YNA − Velocity in water alone.
[2] Values = Velocity in presence of xanthine + YNA − Velocity in xanthine alone.
[3] Values = Velocity in presence of TNA − Velocity in water alone.
[4] Values = Velocity in presence of xanthine + TNA − Velocity in xanthine alone.

hepatoma in that here some idea of the magnitude of the respective A, B, and C patterns was obtained. These patterns, as previously described, were largely qualitative in nature, since they were based on relative velocities of decolorization in the presence and in the absence of the nucleates. Table 7 shows that in the case of liver, under all the conditions noted, the patterns changed from A to B to C with increasing dye concentration, the magnitude at each dye level being shown by the data in comparable columns. At 2.48 mM dye, the pattern of liver in yeast nucleate in the absence of xanthine (second column) was at +52, and in thymus nucleate (sixth column) at +9, thus a C pattern; whereas at the same level of dye in the presence of xanthine the data reveal −10 (fourth column) and −16 (eighth column), respectively, thus an A pattern.

On this basis, the data show that the hepatoma extract with increasing dye concentration was becoming increasingly less C pattern in magnitude. This conclusion, of course, holds only for the range of dye concentrations studied, but it indicates clearly that the effect of the nucleates on the two kinds of tissues was in opposite directions.

The same conclusion may be applied to the data in table 8. These data represent the combined effect of each of the nucleates and xanthine on the change in decolorization velocity of the dye. Here again the effect on the two kinds of tissue was in opposite directions and indicated that the change in pattern in liver was toward increasing, that in hepatoma toward decreasing C.

XANTHINE AND NUCLEATES IN EXTRACTS OF MOUSE LIVER AND OF HEPATOMA

Essentially similar studies to those on rat tissues were performed with normal strain A mouse liver and with the trans-

TABLE 8.—*Effect of xanthine and either YNA or TNA on decolorization velocity in extracts of normal liver and of transplanted rat hepatoma 31 from male Osborne-Mendel rats*

Methylene blue (millimols)	Change in decolorization velocity			
	Xanthine and YNA [1]		Xanthine and TNA [2]	
	Liver	Hepatoma	Liver	Hepatoma
	Minutes^{-1} $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$
0.031		+4, 822		−2, 322
0.062		+3, 277		+1, 372
0.155	−2, 500	+1, 976	−3, 250	+883
0.31	−571	+759	−1, 333	+98
0.62	−47	+217	−238	+19
1.24	+9		−40	
2.48	+72		+66	

[1] Values = Velocity in presence of xanthine + YNA − Velocity in water alone.
[2] Values = Velocity in presence of xanthine + TNA − Velocity in water alone.

planted mouse hepatoma 587. The results are given in table 9. At the lower dye concentration, the pattern was A for the liver and A for the hepatoma in the presence and in the absence of xanthine, whereas at the higher concentration of dye the pattern was A for the liver in the presence and in the absence of xanthine, A for the hepatoma in the presence of xanthine, but C for the hepatoma in the absence of xanthine. Under comparable conditions (table 5), the rat hepatoma possessed a C pattern at each point. Although the mouse hepatoma resembled the rat hepatoma extracts in having a generally lower rate of dye decolorization than that of the respective normal liver extracts, it would appear that at comparable dye concentrations the rate of decolorization was greater in the mouse than in the rat hepatoma; and hence in this respect at least the mouse tumor more nearly approximated its normal tissue of origin than did the rat hepatoma. It might be expected therefore that a C pattern in the mouse tissues would be reached at much higher concentrations of dye than in the corresponding rat tissues. As in the case of the rat tissues, the presence of xanthine required a higher concentration of dye before the pattern changed from A to B to C.

TABLE 9.—*Decolorization velocity of methylene blue in aqueous extracts of normal liver and of transplanted hepatoma 587 from strain A male mice* [1]

Methylene blue (millimols)	Xanthine	YNA	TNA	Decolorization rate	
				Liver	Hepatoma
	Millimols	*Mg.*	*Mg.*	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$
0.62	0	0	0	6,666	909
	0	5	0	2,500	500
	0	0	5	500	72
	1.6	0	0	4,000	666
	1.6	5	0	1,818	303
	1.6	0	5	1,333	152
1.24	0	0	0	135	7
	0	5	0	125	35
	0	0	5	49	14
	1.6	0	0	185	69
	1.6	5	0	145	58
	1.6	0	5	101	40

[1] Aqueous extract of tissues equivalent to 166 mg. per cubic centimeter. Mixtures consisted of 2 cc. extract, 1 cc. dye, and water or solutions of substrates to bring total volume to 5 cc. Temperature, 23° C. Anaerobic conditions maintained throughout by employment of tubes evacuated and sealed at 18 mm. mercury. End point at 98-percent decolorization; pH, 7.1. Concentrations given are of stock solutions.

APPARENT XANTHINE DEHYDROGENASE ACTIVITY IN EXTRACTS OF MOUSE LIVER AND OF HEPATOMA

Table 10 shows that, similar to the case of the rat tissues, the xanthine dehydrogenase activity of mouse liver is a function of the dye concentration as well as of the presence or absence of the nucleates. Again at the lower dye concentration there was a considerable augmentation of activity in the presence of thymus nucleate (columns 6 and 7). Except in the presence of thymus nucleate there was an increase in activity with increasing dye concentration, a phenomenon not generally shown in the case of the comparable rat tissues (table 6).

EFFECT OF THE NUCLEATES ON THE RATE OF DYE DECOLORIZATION IN MOUSE LIVER AND IN HEPATOMA EXTRACTS

The data in table 11, which like those in table 7 reveal the magnitude of the change in pattern of the liver and hepatoma, indicate that the C pattern was attained by the mouse hepatoma at a lower dye concentration in the absence of xanthine than in its presence. In this respect, the data on the rat and the mouse tissues are concordant. The highest dye concentration used for the experiments on the mouse tissues was insufficient to obtain a B or C pattern with liver. However, in contrast with the data in table 7 on rat tissues, the data in table 11 indicate that with increasing dye concentration not only the liver but also the hepatoma became increasingly C in pattern. The data in this table are not sufficiently extensive to warrant further discussion of this point, but they indicate a further similarity between mouse liver and mouse hepatoma which is lacking between rat liver and rat hepatoma. Essentially the same findings are shown, and the same conclusion is reached from the data in table 12.

EFFECT OF VARYING CONCENTRATION OF NUCLEATES ON THE DECOLORIZATION RATE

Several experiments were made of the effect of varying amounts of the nucleates on the decolorization rates of

TABLE 10.—*Apparent xanthine dehydrogenase activity in extracts of mouse liver and of transplanted mouse hepatoma* [1]

Methylene blue (millimols)	Activity in—					
	Water		YNA		TNA	
	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma
	Minutes^{-1} × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4
0.062	−2,666	−233	−682	−197	+833	−86
1.24	+50	+62	+20	+23	+52	−20

[1] Values = Velocity in water + xanthine or in YNA + xanthine or in TNA + xanthine − Velocity in water or in YNA or TNA, respectively.

TABLE 11.—*Effect of YNA and of TNA on decolorization velocity in extracts of mouse liver and of transplanted mouse hepatoma* [1]

Methylene blue (millimols)	Change in decolorization velocity							
	YNA				TNA			
	No xanthine [1]		Xanthine [2]		No xanthine [3]		Xanthine [4]	
	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma
	Min.$^{-1}$×10^4	*Min.*$^{-1}$×10^4	*Min.*$^{-1}$×10^4	*Min.*$^{-1}$×10^4	*Min.*$^{-1}$×10^4	*Min.*$^{-1}$×10^4	*Min.*$^{-1}$×10^4	*Min.*$^{-1}$×10^4
0.062	−4,166	−409	−2,182	−363	−6,166	−837	−2,667	−514
1.24	−10	+28	−40	−11	−86	+7	−84	−29

[1] Values = Velocity in presence of YNA − Velocity in water alone.
[2] Values = Velocity in presence of xanthine + YNA − Velocity in xanthine alone.
[3] Values = Velocity in presence of TNA − Velocity in water alone.
[4] Values = Velocity in presence of xanthine + TNA − Velocity in xanthine alone.

TABLE 12.—*Effect of xanthine and either YNA or TNA on decolorization velocity in aqueous extracts of liver and of transplanted hepatoma 587 from strain A male mice*

Methylene blue (millimols)	Change in decolorization velocity			
	Xanthine+YNA [1]		Xanthine+TNA [2]	
	Liver	Hepatoma	Liver	Hepatoma
	Minutes^{-1} ×10^4	*Minutes*$^{-1}$ ×10^4	*Minutes*$^{-1}$ ×10^4	*Minutes*$^{-1}$ ×10^4
0.062	−4,848	−606	−5,333	−757
1.24	+10	+51	−24	+33

[1] Values = Velocity in presence of xanthine + YNA − Velocity in water alone.
[2] Values = Velocity in presence of xanthine + TNA − Velocity in water alone.

methylene blue in liver and in hepatoma extracts, and it was invariably found that with either sufficiently dilute extracts or sufficiently concentrated dye solutions a maximum at a certain nucleate concentration was attained. This maximum was reached at a lower concentration of thymus than of yeast nucleate. At low concentrations of dye or with relatively high concentrations of extract, this maximum was not observed. Representative data are given in table 13.

At the higher dye concentration the maximum in both liver and in hepatoma was apparently reached at 2.5 mg. of yeast nucleate and at 0.6 mg. of thymus nucleate. At the lower concentration of dye, only the hepatoma extracts revealed a maximum with the nucleates at the respective concentrations mentioned.

It is evident that a B or a C pattern can be achieved in both liver and hepatoma extracts not only by varying the dye concentration but also by varying the nucleate concentration, as well as the degree of dilution of the extract.

EFFECT OF MIXTURES OF THE NUCLEATES

In order to see whether the effects of yeast nucleate and of thymus nucleate were additive when mixtures of the nucleates were employed, the data given in table 14 were obtained. Inspection of these data reveals that there was no summation of effect.

TABLE 13.—*Effect of different concentrations of nucleates on the decolorization rate of methylene blue in aqueous tissue extracts of normal liver or of transplanted hepatoma 587 from strain A mice*[1]

Dye concentration millimols)	YNA	TNA	Decolorization rate	
			Normal liver	Transplanted hepatoma 587
	Mg.	*Mg.*	*Minutes*$^{-1}\times10^4$	*Minutes*$^{-1}\times10^4$
	0	0	137	15
	10	.....	128	63
	2.5	.....	200	65
	.6	.....	170	38
	.15	.....	154	24
1.24	.04	.....	137	21
	.....	10	33	24
	.....	2.5	90	32
	.....	.6	145	29
	.....	.15	132	18
	.....	.04	136	16
	0	0	526	37
	10	.....	222	64
	2.5	.....	357	82
	.6	.....	300	80
	.15	.....	520	58
0.62	.04	.....	520	42
	.....	10	43	17
	.....	2.5	118	30
	.....	.6	271	48
	.....	.15	500	40
	.....	.04	526	35

[1] Mixtures consisted of 1 cc. aqueous extract of normal liver or of transplanted hepatoma 587 (equivalent to 111 mg. tissue), 1 cc. solution (YNA or TNA), and 1 cc. of methylene blue solution. Temperature, 26° C. Concentrations given are of stock solutions.

TABLE 14.—*Effect of mixtures of nucleates on the decolorization rate of methylene blue in aqueous extracts of mouse liver and of mouse hepatoma*[1]

YNA (milligrams)	TNA	Decolorization rate in—	
		Liver	Hepatoma
	Milligrams	*Minutes*$^{-1}\times10^4$	*Minutes*$^{-1}\times10^4$
0	0	164	24
2.5	0	185	75
1.25	1.25	172	58
1.25	0	182	67
0	1.25	169	41
2	.5	178	63
2	0	204	66
0	.5	196	36
0	2	141	35
0.5	0	182	44
0.5	2	130	39
0	2.5	123	33

[1] Mixtures consisted of 1 cc. aqueous tissue extract (equivalent to 111 mg. tissue), 1 cc. solution of YNA or of TNA, and 1 cc. methylene blue at 1.24×10^{-4}M concentration. Temperature, 28° C.

EFFECT OF VARYING CONCENTRATION OF NUCLEATES IN THE PRESENCE OF XANTHINE

In order to see whether a maximum effect could be reached in the presence of xanthine by certain concentrations of nucleates, the experiments described in table 13 were repeated in the presence of xanthine. The data are given in table 15. In no case was a maximum achieved. The effect of the added xanthine was apparently to overcome the accelerating influence of the nucleates at the concentrations described in table 13. It is possible that such a maximum might be attained in the presence of xanthine at sufficiently high concentrations of dye. This would be in accord with the findings described previously in connection with the data in table 5 and figure 2.

TABLE 15.—*Effect of various concentrations of nucleates in the presence of xanthine on the decolorization rate of methylene blue in aqueous tissue extracts of liver or of transplanted hepatoma 587 from strain A male mice*[1]

YNA (milligrams)	TNA	Decolorization velocity	
		Liver	Hepatoma
	Milligrams	*Minutes*$^{-1}\times10^4$	*Minutes*$^{-1}\times10^4$
0	0	149	222
10	.....	118	175
2.5	.....	143	.....
0.6	.....	149	222
0.15	.....	148	.....
0.04	.....	149	222
.....	10	79	89
.....	2.5	118	.....
.....	.6	147	196
.....	.15	148	.....
.....	.04	149	222
0	[2] 0	121	24

[1] Except where noted, mixtures consisted of 1 cc. aqueous tissue extract of either liver or the transplanted hepatoma 587 (equivalent to 111 mg. tissue), 1 cc. either water or nucleate solution (YNA or TNA), and 1 cc. methylene blue dissolved to 1.24×10^{-4}M concentration in 1.6×10^{-3}M xanthine solution. Temperature, 28° C.

[2] Same mixtures as above, except that no xanthine was present, the methylene blue solution being made up in water alone.

TEMPERATURE COEFFICIENT OF THE RATE OF DYE DECOLORIZATION

The data derived from experiments at two different temperatures are given in table 16. For the interval of 10° C., rate increased with increasing temperature by a factor of 1.6–2.6. The larger values of the temperature coefficient were obtained when xanthine was present.

EFFECT OF SALTS ON THE DECOLORIZATION RATE

Data on the effect of the addition of a number of different salts on the decolorization velocity of methylene blue in

TABLE 16.—*Temperature coefficient of the rate of methylene blue decolorization in various mixtures*

Components of mixtures						Rate of decolorization at—	
Extract [1] (cc.)	YNA [2]	TNA [2]	Xanthine [3]	Water	Methylene blue [4]	27° C.	37° C.
	Cc.	*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Minutes*$^{-1}$ $\times 10^4$	*Minutes* $\times 10^4$
1				2	1	49	78
1			1	1	1	118	93
1	1		1		1	93	50
1		1	1		1	72	79
1	1			1	1	69	47
1		1		1	1	28	49

[1] Aqueous extract of strain C male mouse livers (83 mg. tissue per cubic centimeter).
[2] 5 mg. per cubic centimeter.
[3] 1.6×10^{-3}M concentration.
[4] 1.24×10^{-3} M concentration.

mouse liver extracts are given in table 17. Under the experimental conditions chosen in the absence of salts, the extracts revealed a B pattern. In the absence of the nucleates, all the salts used increased the decolorization rate (column 2), bicarbonate being by far the most effective. That the effect of this ion was not simply due to the alkalinity of the sodium bicarbonate used is shown by the data on sodium chloride-sodium hydroxide and sodium hydroxide. The effect was specific for the bicarbonate ion. In the presence of yeast nucleate, phosphate had a slight retarding and fluoride a considerable retarding effect on the decolorization rate, whereas bicarbonate again had an augmenting effect. In the presence of thymus nucleate, chloride, phosphate, and bicarbonate had augmenting effects while fluoride again depressed the decolorization rate. The fact that fluoride had an accelerating effect in the absence of nucleates and a strongly depressant effect in the presence of nucleates is noteworthy. The percentile increase in the augmenting effect of bicarbonate ion was much greater in the absence than in the presence of the nucleates.

TABLE 17.—*Effect of certain salts on decolorization velocity of methylene blue in aqueous extracts of livers from strain A male mice* [1]

Salt	Decolorization velocity in presence of—		
	Water	YNA	TNA
	Minutes^{-1} $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$	*Minutes*$^{-1}$ $\times 10^4$
No salt	[2] 69	125	38
Sodium chloride	[2] 76	125	52
Sodium fluoride	[2] 74	67	27
Monosodium phosphate+disodium phosphate	[3] 78	110	50
Sodium bicarbonate	[4] 286	333	72
Sodium chloride+sodium hydroxide [5]	[4] 79		
Sodium hydroxide [6]	[4] 75	128	35

[1] Mixtures consisted of 1 cc. aqueous extract of liver (equivalent to 111 mg. tissue), either 1 cc. distilled water or 1 cc. nucleate solution (5 mg. YNA or 5 mg. TNA), 1 cc. 0.04M salt, and 1 cc. methylene blue at 1.24×10^{-3}M concentration. Temperature, 27° C.
[2] pH, 7.1.
[3] pH, 7.2.
[4] pH, 7.9.
[5] Sodium hydroxide added to sodium chloride solution so that final mixture gave pH 7.9; sodium-ion concentration in mixture, 0.0105.
[6] In place of 1 cc. salt solution, 1 cc. 0.0026M sodium hydroxide used.

INFLUENCE OF PURINES ON THE DECOLORIZATION RATE

The data on the use of several purines and their derivatives in equivalent concentration in mouse-tissue extracts are given in table 18. In liver extracts the

TABLE 18.—*Influence of purines on the rate of decolorization of methylene blue in aqueous tissue extracts of normal liver from male C3H mice and transplanted hepatoma 98/15 from C3H mice* [1]

Purine	Rate of decolorization in—	
	Normal liver	Transplanted hepatoma 98/15
	Minutes $^{-1} \times 10^4$	*Minutes* $^{-1} \times 10^4$
No purine	130	28
Adenine	111	33
Guanine	222	58
Guanosine	158	38
Adenylic acid	333	67

[1] Mixtures consisted of 1 cc. aqueous tissue extract (equivalent to 111 mg. tissue), 1 cc. saline or saline-purine solution, and 1 cc. methylene blue at 1.24×10^{-3}M concentration. Purine concentration per cubic centimeter: adenine, 0.45 mg.; guanine, 0.51 mg.; guanosine, 1.01 mg.; adenylic acid (yeast), 1.24 mg. Concentration of saline in water and in purine solutions, 0.1M.

effect of added bases, with the exception of adenine, was to accelerate the rate of decolorization, adenylic acid being the most effective. In hepatoma extracts all the bases, including adenine, increased the decolorization rate, adenylic acid again being the most effective. With the exception of adenine, which was not affected, all the bases studied were desaminated in the extracts used (*14*). The residual groups were available for oxidation by the methylene blue provided that the appropriate dehydrogenases were present in the extracts. The dehydrogenation of these bases was studied by Von Euler and Skarzynski (*16*).

STUDIES OF HOMOLOGOUS RAT HEPATIC TISSUES

Four kinds of hepatic tissue are available for study, the normal resting adult liver, regenerating liver after partial hepatectomy, fetal liver, and hepatoma. The last three tissues are growing; but the growth of regenerating liver and of fetal liver is controlled, whereas that of the hepatoma, as far as is known, is uncontrolled. Previous metabolic studies revealed that the fetal liver resembles the hepatoma more than either resembles the adult liver, and that adult liver and regenerating liver are nearly identical (*20*, *21*). Studies of these four types of tissues were extended to the determination of the effect of nucleates on the decolorization rate in extracts of these tissues. The data are given in table 19.

The decolorization rate of regenerating liver was found to be roughly half that of adult resting liver, but both types of tissues possessed an A pattern as far as the effects of the nucleates were concerned. The decolorization rate of fetal liver was very much smaller than that of adult liver but like the latter tissue possessed an A pattern. As mentioned previously, the hepatoma possessed a C pattern, and with the exception of the values for yeast nucleate, the order of magnitude of the decolorization rates approached that of fetal liver. It is of considerable interest that as far as the order of magnitude of the decolorization rates is concerned, hepatoma resembled fetal liver in the absence of added nucleates (water) and in the presence of thymus nucleate, whereas it resembled normal adult liver when yeast nucleate was present (table 19). The latter resemblance is, of course, due to the very considerable accelerating effect which yeast nucleate exerted in the hepatoma extracts. Thus, as far as the present data go, the hepatoma resembles fetal liver in certain respects, adult liver in another respect, and neither tissue in that it has a C pattern as compared with an A pattern for the two normal tissues.

STUDIES ON DIALYZED EXTRACTS OF ADULT LIVER, FETAL LIVER, AND HEPATOMA

Up to this point, all determinations of the decolorization rates were performed with fresh extracts of the tissues. In order to remove the possible

TABLE 19.—*Effect of nucleates on the rate of methylene blue decolorization in aqueous extracts of homologous hepatic tissues of the rat* [1]

Methylene blue (millimols)	YNA	TNA	Decolorization rate of—			
			Adult liver	Regenerating liver [2]	Fetal liver [3]	Hepatoma
	Mg.	*Mg.*	*Minutes*$^{-1} \times 10^4$	*Minutes*$^{-1} \times 10^4$	*Minutes*$^{-1} \times 10^4$	*Minutes*$^{-1} \times 10^4$
0.155	0	0	5,000	----------	83	24
	2.5	0	2,500	----------	53	1,666
	0	2.5	556	----------	25	62
0.62	0	0	714	333	33	5
	2.5	0	667	245	27	370
	0	2.5	294	159	17	23

[1] Mixtures composed of 1 cc. fresh aqueous extract (equivalent to 166 mg. tissue), 1 cc. sodium nucleate solution (YNA or TNA), and 1 cc. methylene blue solution. Temperature, 28° C.
[2] 48 hours after operation.
[3] From rat approximately 2 weeks pregnant.

TABLE 20.—*Dialysis studies A, effect of various substances on the decolorization rate of methylene blue in dialyzed aqueous tissue extracts of normal livers or of transplanted hepatoma 578 in strain A male mice* [1]

Solutions added to extracts and methylene blue [2]						Decolorization rate	
Distilled water (cc.)	Saline [3]	Coenzyme (DPN) [4]	Coenzyme-saline [5]	YNA [6]	TNA [6]	Liver	Hepatoma
	Cc.	*Cc.*	*Cc.*	*Cc.*	*Cc.*	*Minutes*$^{-1}$ × 10^4	*Minutes*$^{-1}$ × 10^4
2						36	24
1	1					105	21
1				1		105	80
	1			1		143	95
1					1	51	33
	1				1	83	60
1		1				36	23
	1	1				2,000	53
		1		1		47	23
		1			1	43	28
			1	1		1,111	53
			1		1	1,000	74

[1] The aqueous tissue extracts, equivalent to 333 mg. tissue per cubic centimeter, were dialyzed 24 hours against distilled water.
[2] Mixtures consisted of 1 cc. dialyzed extract, 1 cc. methylene blue at 0.62×10^{-3}M concentration, and 2 cc. added solutions.
[3] Composition in grams per liter: sodium chloride, 6.8; potassium chloride, 0.4; calcium chloride, 0.2; magnesium sulfate, 0.1; sodium dihydrogen phosphate, 0.125; and sodium bicarbonate, 2.2. The saline is the same as that used by Dr. W. R. Earle for tissue-culture studies.
[4] The diphosphopyridine nucleotide (DPN) was prepared by Dr. J. M. Johnson and was made up at 1.6×10^{-3}M concentration in distilled water.
[5] 1.6×10^{-3}M concentration in saline solution.
[6] 1 mg. per cubic centimeter in distilled water.

TABLE 21.—*Dialysis studies B, dialyzed fetal rat liver* [1]

Solutions added to extract and methylene blue [2]				Decolorization rate
Distilled water (cc.)	Saline [3]	Coenzyme [4]	Coenzyme saline [5]	
	Cc.	*Cc.*	*Cubic centimeter*	*Minutes*$^{-1}$ × 10^4
1				22
	1			64
		1		14
			1	100

[1] Aqueous extract of fetal rat livers (equivalent to 166 mg. tissue per cubic centimeter) dialyzed 24 hours against distilled water.
[2] Mixtures composed of 1 cc. dialyzed extract, 1 cc. methylene blue at 0.62×10^{-3}M concentration, and 1 cc. designated solution.
[3] Composition given in table 20.
[4] Diphosphopyridine nucleotide (DPN), 1.6×10^{-3} M in distilled water.
[5] 1.6×10^{-3} in saline.

influence of dialyzable substrates and salts, studies were made on dialyzed tissues.[7] Inasmuch as an important dialyzable component of the tissue dehydrogenase is coenzyme, particular attention was paid to this substance. A sample of diphosphopyridine nucleotide (DPN) was provided by Dr. J. M. Johnson. The effect of salts was likewise investigated. The data on the tissues are given in tables 20 and 21.

[7] The salt solution used, referred to as saline, is that of Earle (*22*), without added dextrose.

The decolorization rate of the dialyzed tissues was considerably lower than that of the fresh tissues. Coenzyme added to the dialyzed extract not only did not increase the decolorization rate but actually depressed it. Addition of salt solution alone caused a considerable increase in the decolorization rate; and when coenzyme was further added with the salt, a very considerable increase in rate was observed. It is evident that for the accelerating effect of coenzyme to manifest itself, the presence of salts is necessary. The presence of either of the nucleates depressed by half the decolorization rate of liver extracts plus coenzyme-saline but did not affect the rate of the hepatoma extracts under the same conditions.

EFFECT OF ADDED SALTS ON COENZYME IN DIALYZED EXTRACTS

The fact (table 20) that salts are necessary for the action of coenzyme in dialyzed extracts led to studies directed toward the nature of the specific ion or ions in the salt mixture responsible for this effect. Each of the salts was prepared separately in the concentration in which it occurred in the saline solution (table 20) employed.

Since the bicarbonate alone is alkaline in reaction, a solution of sodium hydroxide at the same pH of the bicarbonate, 8.5, was prepared in order to have a proper control. The mixtures consisted of 1 cc. dialyzed A mouse liver extract (approximately 166 mg. tissue), dialyzed against distilled water 3 days, 1 cc. salt solution, 1 cc. coenzyme solution at 0.8 x 10^{-3}M concentration in water, and 1 cc. methylene blue at 0.62x10^{-3}M. The data are given in the following tabulation:

Salt:	*Decolorization rate of methylene blue (Minutes^{-1} × 10^4)*
No salt	21
Saline	83
Sodium chloride	31
Potassium chloride	25
Calcium chloride	25
Magnesium sulfate	21
Sodium dihydrogen phosphate	25
Sodium bicarbonate	166
Sodium hydroxide	21

It is evident that although sodium chloride possessed a slight effect in increasing the decolorization rate due to the coenzyme, the bicarbonate ion had by far the greatest effect. The similarity of the present findings on the effect of bicarbonate on dialyzed tissue extract plus coenzyme to the findings on effect of this ion on the fresh extract (table 17) was not unexpected. The dependence on coenzyme activity and the presence of bicarbonate ion was, however, unexpected and striking. The complete salt mixture had a smaller effect on augmenting the decolorization rate than had the bicarbonate alone. It was as if the presence of the other salts in the mixture depressed somewhat the effect of the bicarbonate. The necessity for the presence of bicarbonate for respiration was pointed out by Warburg (*23, p. 129*).

The fact that the dialyzed tissues in the absence of any added material still decolorized methylene blue, even after some time, led to the suspicion that there may be some nondialyzable substrate present in these tissues. Previous workers using dialyzed tissues had apparently broken off their experiments with such tissues too soon, generally at 5 hours. The first possible nondialyzable substrate in hepatic tissues which comes to mind is glycogen. Experiments with this material were therefore set up. The results are given in table 22.

TABLE 22.—*Effect of glycogen on the decolorization rate of methylene blue in dialyzed mouse-liver extracts* [1]

Solutions					Decolorization rate
Glycogen (cc.) [2]	Distilled water	Saline [3]	Coenzyme [4]	Coenzyme-saline [5]	
	Cc.	*Cc.*	*Cc.*	*Cc.*	*Minutes^{-1}* × 10^4
	1		1		21
		1	1		83
1			1		77
1	1				61
1				1	400
1		1			250

[1] Mixtures consisted of 1 cc. dialyzed mouse-liver extract (approximately 166 mg. tissue) dialyzed against distilled water for 3 days, 2 cc. of the designated solutions, and 1 cc. methylene blue at 0.62 × 10^{-3}M concentration.
[2] At 1-percent concentration in water. The decolorization rate with a solution of glycogen dialyzed against distilled water for 24 hours was approximately 10 percent lower than with a nondialyzed solution.
[3] Composition given in table 20.
[4] At 0.8 × 10^{-3}M concentration DPN in water.
[5] At 0.8 × 10^{-3}M concentration DPN in saline.

It is clear that the presence of glycogen alone in the dialyzed extract produced an acceleration in the decolorization rate, that this rate was still further increased in the presence of salt solution, and even still further increased when coenzyme as well as salt was present. Whether the glycogen functioned as such in these phenomena or whether degradation products of this polysaccharide were responsible is not entirely answered at the present time. Liver contains a highly active glycogen-degrading system (*21*).

GLYCOGEN AND NUCLEATES

The effect of the nucleates on decolorization rates of the dye in mixtures of extract, coenzyme-saline, and glycogen was studied. The data are presented in table 23. The extract had been dialyzed only 1 day as compared with that described in table 22, which had been dialyzed for 3 days.

Under the conditions here presented the addition of glycogen resulted in a retardation of the decolorization rate;

TABLE 23.—*Glycogen and nucleates in extracts of dialyzed mouse liver* [1]

Solutions					Decolorization rate
Glycogen [2] (c c.)	Distilled water	Coenzyme-saline [3]	YNA [4]	TNA [4]	
	Cc.	*Cc.*	*Cc.*	*Cc.*	*Minutes*$^{-1}$ $\times 10^4$
	2	1			320
1	1	1			233
1		1	1		277
1		1		1	166
	1	1	1		250
	1	1		1	93

[1] Mixtures consisted of 1 cc. dialyzed mouse-liver extract (approximately 166 mg. tissue) dialyzed against distilled water 1 day, 3 cc. of the designated solutions, and 1 cc. methylene blue at 0.62×10^{-3} M concentration.
[2] At 1 percent in water.
[3] At 0.8×10^{-3} M concentration diphosphopyridine nucleotide (DPN) in saline solution.
[4] At 0.5 percent in water.

but when not only glycogen but also the nucleates were added, the relative decolorization rate was increased. Thus, these data are remindful of those given for xanthine dehydrogenase activity (table 6). The apparent activity for this substrate (at low dye) was negative in sign in the absence of nucleates but positive in sign in the presence of the nucleates. The relative order of magnitude of this activity was greater for thymus than for yeast nucleate. Similarly when glycogen was used, the sign for the activity with this substrate was negative, but in the presence of the nucleates the sign was positive, being more strongly positive in the presence of thymus than of yeast nucleate.

DISCUSSION

This study is essentially exploratory in nature. As stated in the introduction, the results presented are in no sense complete. No attempt was made to follow in detail the reactions which were measured by the rate of decolorization of methylene blue. Our concern was to observe empirically the effects consequent upon the addition of certain naturally occurring substances to the more or less arbitrarily established systems as found in the cell extract. It is conceded that such systems quite certainly depart to an unknown and at present a largely unknowable extent from the living intracellular systems from which they are derived. Such defect is, however, inherent in any in vitro study. It is also considered very probable that any over-all effects of such magnitude as to be readily and reproducibly measured, arising as a consequence of experimental changes in these arbitrary systems, reflect at least qualitatively the effects of similar changes in the intracellular systems of origin. With these qualifications, the results may be considered.

The over-all activity of the dehydrogenase system in the tissue extract depends upon the concentrations of (1) acceptor (methylene blue), and (2) substrates and enzymes (tissue extract). Even when very thoroughly dialyzed tissue was used (table 23), a definite residual activity was manifested in the tissue. In the instance given, the decolorization in the system extract plus methylene blue at 0.62×10^{-3} M was complete in 8 hours. It is to be regretted that much experimental work, particularly that of an exploratory type, has been done in which no marked change in color in 5 hours was taken as zero change. Our experience shows that many significant data have probably been overlooked in this way. If we had adopted such criteria, the definite and rather surprising delaying action of coenzyme shown in table 21 would not have been noted. Von Euler and Skarzynski (*16*) reached the conclusion that thymonucleate had no effect in extracts of dialyzed rat liver on this basis. The present results demonstrate the error of this arbitrary limitation of time.

The two nucleates exercised a very definite effect on the over-all dehydrogenase activity in all tissues tested. This effect varied in sign, dependent upon the conditions of the experiment, and the results obtained were for given concentrations of acceptor, nucleate, and extract qualitatively, and sometimes quantitatively predictable (providing only that the source of the extract was known and had been subjected to some preliminary study.

There are interesting and suggestive differences between the action of the two nucleates throughout. Thymus nucle-

ate retarded more or stimulated less the over-all dehydrogenase activity. Its general effect as compared with yeast nucleate was conservative. The thymus nucleate also showed a definite stimulating effect on the activity of xanthine oxidase at low dye concentrations.

As to the action of the nucleates, it is not possible to prove conclusively from the data so far obtained whether this is catalytic, metabolic, or a combination of both.

The studies carried out on the nucleodesaminases (*14*) showed conclusively that the acids exposed in contact with similar tissue extracts and under much more severe conditions are not split and are only very slowly desaminated. Thus, under the conditions of the present experiments, there appear no obvious points of attack for dehydrogenase action on the nucleates. Further if the only action found, that of desamination, did occur during these experiments, the hydrogen transfer thus made possible, taking into consideration the acceptor concentration and that of the nucleates, may be too small to account stoichiometrically for the increased reduction of the acceptor observed. Table 20 indicates that the activity of dialyzed mouse tissues was raised by added nucleates and was raised still further by the addition of a saline solution. The stimulating effect of this solution was shown to be almost entirely due to its bicarbonate content. If the desamination of the nucleates played any part in the effect, bicarbonate should have acted in the opposite sense to that found, since it depresses desamination (*14*). Also as pointed out, the effect was retarding in most instances and was not (table 14) additive for the two nucleates.

It seems reasonable to consider that the added nucleates were not metabolized to any considerable extent in these experiments. There is therefore some evidence that they influenced the course of the reactions observed but appeared essentially unchanged, i. e., their action was catalytic. A favorite hypothesis often adopted in these circumstances is to consider that an acceptor-enzyme complex is entered into and the rate of its formation and disruption affected by the factor considered (in this case the nucleate). The over-all activity would then depend on the concentrations of all the components. As an alternative hypothesis, it may be assumed that the nucleates compete either as substrates or as hydrogen-transport systems with the usual coenzymes for dehydrogenases. More data are needed for conclusive decisions.

It must be stressed that the data are clear-cut and exhibit an internal consistency. As shown in figure 2 and as can be shown for the other data, if in any given system only the acceptor (dye) concentration is varied, there is a straight line relation between the logarithm of the acceptor concentration and the logarithm of the rate (reciprocal of the decolorization time). The slope of the curves and their position in the coordinate system (fig. 2) depend on the nature of the constituents and their relative concentrations and thus give rise to the patterns of response A, B, C previously noted. There is thus manifest an underlying orderliness in the phenomena, but much more work must be done before even a tolerably complete mathematical treatment can be attempted.

Apart from this only partially successful attempt at analysis, the clear demonstration of the effects of ions, and in particular the major effect of the bicarbonate ion on the activity of diphosphopyridine nucleotide (coenzyme I), is of considerable interest. It would appear that in the systems investigated the bicarbonate ion is indispensable for the action of the nucleotide (tables 17 and 22). While, as before noted, the need for this ion in respiration was observed by Warburg, this is, we believe, the first direct evidence that it is essential for the activity of the coenzyme in dehydrogenase systems. The effect of this ion on thymonucleodesaminase (desoxyribose) found by Greenstein and Chalkley (*14*) is of some interest, since the effect is opposite to that on the dehydrogenase activity.

Attention is drawn to the residual dehydrogenase activity in tissue ex-

665190—45——4

tracts after dialysis, and the continued response of this residual activity to addition of nucleates, and salts (table 20). If it is assumed, according to the evidence presented, that the nucleates are essentially catalysts rather than subtrates, then the results obtained may indicate that enzymes acting on nondialyzable as well as dialyzable substrates are affected by nucleates, both in the normal mouse liver and in transplanted mouse hepatoma tissue. Other tissue would undoubtedly give a similar picture. The pattern of the lessened effect of thymus as contrasted with yeast nucleate holds. These facts place a serious obstacle before further attempts to carry on the research with the simple technique thus far used. It is evident that in these studies the apparent activity of a dehydrogenase for any substrate is not a true index, except where all other substrates and enzymes present in the system are controlled or known. Dialysis makes this control possible to a reasonable degree only for salts, dialyzable substrates, and the coenzymes; but the occurrence of activity of the nucleates upon undialyzable substrates necessitates the control of these also before the analysis can be carried further. No adequate techniques for their segregation could be devised at this time. We are, however, considering an attempt to overcome this obstacle at least partially. For it is apparent (table 20) that addition to the dialyzed extract of bicarbonate alone produced a very great increase in dehydrogenase activity. Under such conditions, it is hard to see how small molecule substrates could be present unless perchance by some unknown mechanism bicarbonate effected their production from compounds of very great molecular volume. Barring this, the dehydrogenation of large molecules is foreshadowed. In either event, the need for at least an attempt to carry the research forward is urgent.

The single, readily available, nondialyzable substrate that can reasonably be assumed to be present in relatively large amount in the liver is glycogen, and the effects consequent upon its addition to the systems studied were shown in tables 22 and 23. As noted, the general effect of the nucleates was evident, and both coenzyme and salt solution added to the system containing glycogen greatly raised its activity; but as pointed out, no conclusion could be reached whether the effect, insofar as glycogen is concerned, was due to the split products or to the complete molecule. Further it was shown (table 23) that under certain conditions the addition of glycogen retarded the dehydrogenase activity. The value of this slight study of a nondialyzable substrate seems to the authors to be in the suggestion it holds that the action of the nucleates is so widespread and far-reaching in the complex chemical machinery of the cell that it may well be taken as a fundamental factor therein, especially as this effect is so marked on the primary step in the oxidative mechanism (dehydrogenation).

The differences between the activities of systems derived from different tissues point to a specificity of chemical structure probably correlated with the role of the tissue of derivation within the organism. It is too early to generalize as to the differences found between the extracts derived from tumor and normal tissues. It must be stressed that these differences are established only for the tissues used, and implications suggested thereby should not be given undue weight.

Freshly prepared extracts from both rat and mouse hepatoma tissue showed a very much lower dehydrogenase activity than did similar extracts from the homologous normal-liver tissues. This fact suggests that the tumorous tissue may have either less active enzymes or less readily available substrate, or both. That there is a definite lack of dialyzable substrate in the tumor as respects the normal homologue is definitely shown by comparison of the dehydrogenase activities in the fresh and dialyzed extracts from the two types of tissues in the mouse. (Unfortunately no rat hepatoma was available for dialysis experiments.) A general comparison of the respective

activities of normal mouse liver and hepatoma as shown in the fresh extracts is readily made from tables 9, 13, 14, and 18. It shows that on the average and under the various conditions used, the activity of the normal tissue is of the order of 10 times that of the hepatoma, while after dialysis (shown by table 20) the activities in extracts of both tissues are of the same order, namely, that of the hepatoma, If the activities of extracts of the rat and mouse hepatomas are compared at equal methylene blue concentration (0.62 mM), it is at once apparent that they are of the same order of magnitude. When the changes on dialysis, just discussed, are taken into consideration, the facts indicate very strongly that both the rat and mouse tumors are greatly deficient in dialyzable substrates, i. they are empty tissues closely resembling dialyzed normal liver as respects substrate content. Although these tumors are low in coenzyme (*24*), they differ from the dialyzed normal tissues as to salt content, in that they are higher. Further experimentation with this in mind should be fruitful. This relatively low dehydrogenase activity, ascribable to lack of substrates, is not specific for tumor tissue alone as is indicated in table 19. Extracts of rat hepatoma and of rat fetal liver showed a relatively low activity as compared with extracts of normal rat liver; the regenerating liver did not. We are, therefore, barred from the assumption that such deficiencies as may be ascribed to substrate are merely correlates of rapidly proliferating tissues. The regenerating liver was examined at the height of regenerative activity (48 hours after hepatectomy) and was fully comparable proliferatively with the hepatomas.

Attention is called to a difference (table 19) shown in the behavior of the dehydrogenases in four homologous hepatic tissues of the rat, adult, fetal, regenerating, and neoplastic. On addition of thymus nucleate, the activity in the adult, regenerating, and fetal tissues was lowered, but it was definitely increased in the neoplastic tissue. On addition of yeast nucleate it was depressed in adult, regenerating, and fetal tissue, but very greatly increased in the neoplastic. This difference held over a fairly wide range of acceptor concentration. Compared thus, the neoplastic hepatic tissue differed from the normal adult, fetal, and regenerating hepatic tissues in that it responded positively in dehydrogenase activity to addition of nucleates (a C pattern) while the others responded negatively (an A pattern). The most notable response by the neoplastic tissue was to the yeast nucleate. The activity values attained in the presence of yeast nucleate corresponded closely (i. e., were definitely of the same order) to those given on a similar addition to an extract of the normal adult tissue. As a result of this similarity in values, the activities appear statically alike. If the relatively slight differences in order of magnitude are set aside, there appear certain groupings of the activities. The order of magnitude of the dehydrogenase activity in these extracts in response to thymus nucleate is of the same low order in fetal and neoplastic and of the same high order in regenerating and adult liver. In respect to addition of yeast nucleate, this order of magnitude is high in the hepatoma, regenerating, and normal adult tissues, and low in fetal tissue.

If it can be assumed that the activities are correlated with the region of the cell for which the nucleates are characteristic, these resemblances and groupings suggest that response to the cytoplasmic (ribosenucleate) component is of the same order in adult, regenerating, or neoplastic tissue but is lower in fetal tissue, whereas the fetal and neoplastic tissues resemble each other more in response to nuclear activity. Does the neoplastic hepatic cell differ from the adult in that its response to the nucleus has approached more nearly that of the embryonic cell nucleus, while its cytoplasmic response has remained nearly normal? Some such state as this seems indicated in so far as dehydrogenase activity is concerned.

SUMMARY

Studies were made of the effect of added yeast nucleate and of added thymus nucleate on the decolorization rate of methylene blue in tissue extracts treated in various ways. The effect of the added nucleate was either to depress or accelerate the decolorization rate, and the direction which this effect took depended upon (1) the concentration of the dye, (2) the degree of dilution of the extract, (3) the concentration of the nucleate, (4) the nature of the nucleate, (5) the nature of the tissue, and (6) the addition of oxidizable substrates to the extracts. These phenomena were observed with a modified type of procedure.

At relatively low dye concentrations, the addition of both nucleates resulted in a depressing of the decolorization rate, at relatively high dye concentrations the nucleates produced an acceleration in the decolorization rate; at intermediate concentrations of the dye, yeast nucleate accelerated, whereas thymus nucleate depressed the decolorization rate. When liver and hepatoma were compared under nearly identical conditions, the hepatoma in contrast with the liver revealed an accelerating effect by both nucleates over a wide range of dye concentrations. In the magnitude of the effect produced by yeast nucleate, hepatoma resembled adult normal liver, whereas in the magnitude of the effect produced by thymus nucleate, it resembled fetal liver.

In the presence of added oxidizable substrate, such as xanthine, the point at which the effect of added nucleates changed from a depressing to an accelerating one was shifted in the direction of higher dye concentrations. In accord with this was the finding that with increasing dilution of the extract (i. e., less substrate) the effect of nucleates became accelerating on the decolorization rate of the dye.

With increasing concentration of the nucleates under otherwise constant conditions, the depressant effect on the decolorization rate increased, thymus nucleate producing by far the greater effect. With decreasing concentrations of the nucleates and in the absence of added substrate, a maximum accelerating effect was attained with each nucleate.

Xanthine dehydrogenase activity was calculated in several of the mixtures and found to be a function of the dye concentration, of the xanthine concentration, and of the presence or absence of nucleates. At low concentrations of dye, the activity had a negative sign in water, but in the presence of thymus nucleate this activity was strongly positive. The nucleates appeared to augment xanthine dehydrogenase activity.

Coenzyme I (diphosphopyridine nucleotide) was a component in the tissue extracts responsible in considerable measure for the decolorization of methylene blue, but it was found that this factor was active only in the presence of the bicarbonate ion. This finding of the effect of bicarbonate was drawn from studies of dialyzed as well as of fresh tissues. No other ion in a salt mixture approached the bicarbonate effect, and in the absence of this ion added coenzyme produced a retarding effect on the decolorization rate.

The ultimate decolorization of dialyzed extracts led to a study of possible nondialyzable substrates, and it was found that glycogen added to such extracts accelerated the decolorization rate of the dye.

The implications of these findings are discussed.

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EFFECT OF SODIUM BICARBONATE, PYRUVATE, AND DIPHOSPHOPYRIDINE NUCLEOTIDE (DPN) ON DEHYDROGENASE ACTIVITY

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INTRODUCTION

The over-all dehydrogenase activity, as measured by the rate of decolorization of methylene blue, is very much lower in dialyzed than in freshly prepared tissue extracts. This difference is due, to a considerable extent, to the loss of coenzyme factors, including the pyridine nucleotides, from the dialyzed extracts. The authors (*1*) have shown that an activity approximating that of fresh-mouse-liver extract can be restored to a dialyzed extract of this tissue by the addition of both diphosphopyridine nucleotide [1] and sodium bicarbonate. The addition of bicarbonate alone to the dialyzed extract results in a definite but relatively small stimulation in activity; the addition of DPN alone results in a definite retardation in activity. Only when bicarbonate and DPN are added together to the dialyzed extract does a striking rise in dehydrogenase activity occur.

The problem of the utilization of carbon dioxide by tissues is an old one. In plants, this utilization is concerned with the phenomena of photosynthesis. In heterotrophic bacteria, Werkman and Wood (*2*) showed that carbon dioxide participates in certain dissimilation reactions. In animal tissues, many investigators have reported that at least one of the reactions in which carbon dioxide participates is that with pyruvic acid to form oxaloacetate (*3–6*). The techniques of these studies involved chiefly those of manometry and of the use of radioactive and heavy isotypes of carbon. The studies described in the present paper extend the dehydrogenase investigations (*1*) to dialyzed liver extracts containing pyruvate as well as bicarbonate and DPN.

[1] Hereinafter referred to as DPN.

EXPERIMENTAL PROCEDURE

The materials used and the methods of preparing the tissue extracts and of measuring the dehydrogenase activity of the mixtures employed are described in the preceding paper (*1*). The pyruvate was a purified specimen of the sodium salt, prepared by Dr. Robertson, formerly of this Institute. The sodium bicarbonate was prepared by bubbling commercial carbon dioxide through a sodium hydroxide trap into a sodium hydroxide solution of known normality. No essential difference, however, was observed between the behavior of such a preparation and that of a chemically pure commercial specimen of sodium bicarbonate, in the systems studied.

Mixtures of the tissue extract with the components designated were prepared in replicate, the methylene blue being placed in the side arm. The dehydrogenation tubes were evacuated at 18 mm. mercury for 2 minutes and sealed. The dehydrogenation tubes were divided into two groups: one group was composed of mixtures into which the dye was tipped immediately after evacuating and sealing the tubes, and the other was composed of identical mixtures into which the dye was tipped 2 hours after evacuating and sealing the tubes.

RESULTS

Several experiments were performed on various mixtures with entirely concordant results. Representative data are given in table 1.

As in previous findings, it was noted that the addition of bicarbonate to the dialyzed extract resulted in a definite stimulation, and the addition of DPN in a definite retardation, in activity, whereas addition of both bicarbonate and DPN simultaneously resulted in a very considerable stimulation in activity, to a value approximating the ac-

TABLE 1.—*Effect of sodium bicarbonate, DPN, and pyruvate on the decolorization rate of methylene blue in dialyzed extracts of mouse liver*[1]

Components of mixtures[2]	Amount	Decolorization rate after standing[3]—	
		0 hours	2 hours
	Cc.	*Minutes*$^{-1}$ x 10^4	*nutes*$^{-1}$ x 10^4
Sodium chloride-sodium hydroxide	1	[4] 10	9
Water	1		
Sodium bicarbonate	1	[5] 24	24
Water	1		
Sodium chloride-sodium hydroxide	1	5	5
DPN[6]	1		
Sodium bicarbonate	1	840	840
DPN[6]	1		
Sodium chloride-sodium hydroxide	1	11	11
Pyruvate	1		
Sodium bicarbonate	1	[7] 24	33
Pyruvate	1		
Sodium bicarbonate-DPN	1	[8] 385	[9] 1,000–3,000
Pyruvate[10]	1		

[1] Aqueous extract of mouse liver (equivalent to 111 mg. tissue per cubic centimeter) dialyzed 48 hours at 0° C. against distilled water.

[2] Stock solutions as follows: sodium bicarbonate, 0.04M; DPN, 1.12 mg. per cubic centimeter of water; sodium pyruvate, 1.6 x 10^{-2}M; sodium chloride-sodium hydroxide, 0.04M in respect to sodium ion and containing enough sodium hydroxide to give the same pH, namely, 8.0, in the mixture containing tissue extract, as do the same mixtures with sodium bicarbonate; bicarbonate-DPN, 1.12 mg. of the nucleotide per cubic centimeter 0.04M sodium bicarbonate; methylene blue, 0.31 x 10^{-3}M. Solutions in dehydrogenation vessels composed of 1 cc. tissue extract, 1 cc. methylene blue solution, and the volumes of components designated, a total of 4 cc. in each case. Temperature, 26° C. Technique of measurement according to (*1*). The pH of the mixtures did not vary by more than 0.1 unit during the course of the reactions.

[3] "0 hours" refers to tubes into which dye was tipped immediately after sealing; "2 hours" refers to tubes into which dye was tipped 2 hours after sealing. Temperature during this interval, 26° C.

[4] Rate under these conditions for freshly prepared mouse-liver extract was approximately 300.

[5] Rate under these conditions for freshly prepared mouse-liver extract was approximately 1,100.

[6] At the end of a 3-hour period of incubation of DPN with liver extract at pH 8.0, the mixture was examined for increase of ammonia. The ammonia found corresponded to a desamination of DPN amounting to roughly 5 percent of available amino groups. The metabolism of DPN, therefore, during the few minutes required to decolorize the methylene blue was certainly negligible.

[7] With 0.04M and with 0.4M pyruvate, the values were 45 and 60, respectively.

[8] With 0.4M pyruvate in this mixture, the value was 530.

[9] With 0.04M pyruvate in this mixture and after 5 hours of incubation before the methylene blue was tipped in, the value was 5,000.

[10] Addition of 0.001M manganese (Mn^{++}) was somewhat retarding.

tivity of the fresh tissue. Incubation of these various mixtures for 2 hours before adding the dye produced little if any change in the activity of each mixture. Addition of pyruvate to the dialyzed extract did not alter the activity, nor did the activity change when the mixture was allowed to stand 2 hours before adding the dye. Addition of bicarbonate and pyruvate to the dialyzed extract brought about a stimulation in activity of a magnitude characteristic of that due to the bicarbonate alone, but a similar mixture allowed to stand 2 hours before adding the dye showed a relatively slight but definite augmentation in activity. When bicarbonate, DPN, and pyruvate were added to the dialyzed extract, there was a considerable increase in activity, which, nevertheless, was not so great an increase as that shown by the mixture of bicarbonate and DPN. It was as if the addition of pyruvate under these conditions reduced the activation which a mixture of bicarbonate and DPN would otherwise show. However, when mixtures of bicarbonate, DPN, and pyruvate were allowed to stand 2 hours before the dye was added, a most striking increase in dehydrogenase activity was noted. Further experiments revealed that the magnitude of this increase in activity was greater with higher concentrations of pyruvate and with longer periods of incubation.

DISCUSSION

So far as the authors are aware, the present studies on the effect of the substances employed in dehydrogenase investigations of dialyzed animal-tissue extracts constitute a new approach and suggest a relatively simple means of demonstrating carbon dioxide utilization by such tissues. The work of others (*3–6*) justifies the assumption that the rise in activity shown on the addition of bicarbonate, pyruvate, and DPN had its origin in the phenomenon of carbon dioxide fixation in the dissimilation of pyruvate, resulting in the production of substrates, the dehydrogenation of which was catalyzed by DPN. The rise seen on addition of bicarbonate and DPN alone is another story for (1) some dehydrogenation occurred on addition of bicarbonate alone as evidenced by a 140–150 percent increase in decolorization rate, (2) the enormous increase in this rate, some 8,000 percent on the further ad-

dition of DPN, was not increased after standing, as was the increase on addition of pyruvate, and (3) the substrates oxidized must have been nondialyzable prior to the addition of the reactants.

It would thus appear that in addition to the suggestion of the carbon dioxide fixation with pyruvate, there was shown a dehydrogenation activity with nondialyzable substrate, for which bicarbonate was needed and which was strongly furthered by DPN. It is quite probable, however, that the DPN in this instance was not involved in the production of oxidizable substrate but was simply acting as usual as a transfer agent between the substrate developed through the bicarbonate action on the nondialyzable substance or substances and the acceptor (methylene blue).

It seems possible that the complete inactivation of dehydrogenase activity in cells, observed by Hes (7), in the absence of carbon dioxide may arise not alone from the need for carbon dioxide for the dissimilation of low-molecular-weight substrates, but also from its need to permit dehydrogenase activity subserved by nondialyzable (high-molecular-weight) substrates. The nature of these substrates is still to be revealed.

SUMMARY

The over-all dehydrogenase activity, as measured by the rate of decolorization of methylene blue, was determined under controlled conditions for mixtures of dialyzed mouse-liver extracts with sodium bicarbonate, diphosphopyridine nucleotide (DPN), and pyruvate. These rates were studied in mixtures to which dye was added immediately after evacuating and sealing the tubes, and in mixtures allowed to stand 2 hours after evacuating and sealing the tubes, before the dye was added.

Addition of bicarbonate alone had a stimulating effect, while the addition of DPN alone had a retarding effect, but the addition of both had a very considerable stimulating effect. Addition of pyruvate had no effect on the activity, addition of bicarbonate and pyruvate had the same stimulating effect as had bicarbonate alone, and the addition of bicarbonate, DPN, and pyruvate had a decided stimulating effect, which was, nevertheless, smaller in magnitude than that shown in mixtures of bicarbonate and DPN.

After standing 2 hours before the dye was added, none of the mixtures showed any change in activity except that of bicarbonate and pyruvate, which was slight, and that of bicarbonate, DPN, and pyruvate, which was enormous.

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